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Investigation of derivatives of Δ^3-carene

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ABSTRACT

The reaction of Δ^3-carene, under different conditions, to form the nitrosochloride compound has been studied. The yield never exceeded 15 %. The prepared oxime (m.p. 97–98°) is optically active $[\alpha]_D^{25} = +374°$ which will exclude equilibrium with a seven-membered ring structure. The oxime, when hydrolysed over dilute H_2SO_4 or oxalic acid, is converted by the loss of two hydrogens to 3,4-dimethylacetophenone.

Permanganate oxidation of the oxime at 0°C gives an optically active acid $C_8H_{12}O_4$, m.p. 115–117°, $[\alpha]_D^{25} = +62°$ giving a methyl ester $C_{10}H_{16}O_4$, (b.p. 104–106°/10 mm Hg), $[\alpha]_D^{25} = +50°$. When hydrolysed, this ester gives an optically inactive acid which is found to be identical with the acid Owen and Simonsen have reported to be *cis*-homocaronic acid. This latter acid must, however, be 3,3-dimethylhexene-1,6-dioic acid, since when oxidized by permanganate it gives β,β-dimethylsuccinic acid and is reduced (H_2; PtO_2) to β,β-dimethyladipic acid. The same conversion is also obtained on heating the optically active acid with Ac_2O in a sealed tube at 210°.

When heated at 100° with hydrochloric acid, the optically active acid is converted to optically inactive terpenylic acid, which, in combination with the conversions described, gives very strong evidence for the optically active acid to be the real homocaronic acid.

All the reactions performed in this investigation support the correctness of the formula for Δ^3-carene given by Simonsen.

Δ^3-carene (I) is a constituent of Swedish turpentine from *Pinus sylvestris* of which few derivatives are known. This is partly explained by the relative difficulty in obtaining Δ^3-carene pure for synthesis, but the main reason is certainly due to the reactivity of the *cyclo*propane ring. In earlier communications, the present author has studied the analysis and purification of Δ^3-carene from Swedish sulphate turpentine (1, 2), and since certain derivatives of Δ^3-carene are of special interest for the chemical understanding of turpentine eczema, this investigation was begun.

Δ^3-carene nitrosochloride and oxime

One of the reactions of olefines commonly used in terpene chemistry is the addition of nitrosochloride to the double bond. This compound of Δ^3-carene was prepared by M. Lagache (3) in 1927, but since that time the compound has been reported only once in the chemical literature (4). Lagache has used the ethyl nitrite method to synthesize Δ^3-carene nitrosochloride (II) and has also prepared the corresponding oxime. The present author has found the purity of Δ^3-carene to be of importance for a successful performance of the reaction using the composition given by Lagache, and only pure Δ^3-carene gave a crystalline product on synthesis. Since the purified

carene sample was available only in small quantities, methods were investigated which could give a satisfactory result when an impure Δ^3-carene was used as starting material. Tables 1 and 2 demonstrate different conditions under which test reactions have been run. The yield has never exceeded 15 per cent. The nitrosochloride reaction belonged unfortunately to the unpredictable ones, and of around forty syntheses performed at this laboratory, four have not given any crystalline products, and in four cases sudden decompositions occurred.

The alkaline conversion of Δ^3-carene nitrosochloride to the optically active oxime in alcoholic solution, according to Lagache (3), was successful only when it was performed rapidly on a small scale. It was found possible, by crystallization from light petroleum, to increase the melting point of the oxime to a higher value than given by Lagache.

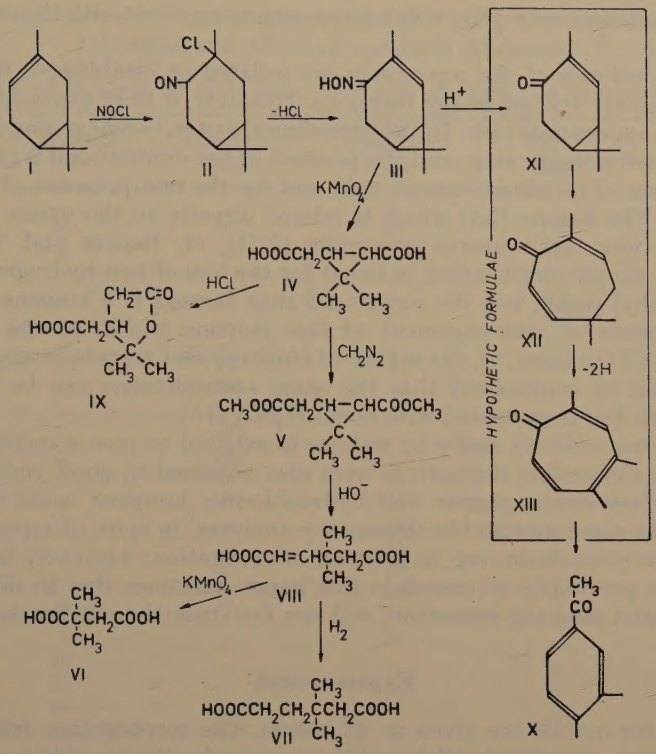
Configuration of Δ^3*-carene oxime*

The configuration of carene oxime (III) is of interest, since many conversions could occur on removal of hydrogen chloride from the nitrosochloride. Limonene and sylvestrene derivatives may be formed, but also ring opening to yield seven-ring compounds may occur in conformity with the formation of eucarvone. Hydrogen chloride could be split off to form a semicyclic double bond as in α-pinene oxime, or to give a cyclic double bond as in limonene oxime.

Ozone reaction on carene oxime established that no semicyclic double bond was formed, and the experiment was checked with a simultaneous test with pinene oxime, cf. Ruzicka and Trebler (5). However, attempts to isolate crystalline products of the ozone reaction failed. Permanganate oxidation in ice-cold solution gave an optically active, crystalline acid $C_8H_{12}O_4$ (IV) which was esterified by diazomethane to the liquid ester $C_{10}H_{16}O_4$ (V). The IR-spectrum of this optically active ester was not identical with those of the inactive dimethyl esters of the *cis*- or *trans*-homocaronic acids synthesized according to Owen and Simonsen (6). However, when the optically active ester (V) was hydrolysed by sodium hydroxide solution, an optically inactive acid was formed which was found to be identical with Simonsen's *cis*-homocaronic acid. This conversion also occurred when the acid (IV) was heated with acetic anhydride.

This discovery suggested that the configuration of Simonsen's *cis*-homocaronic acid was incorrect. Permanganate oxidation of this acid, under the mild conditions given above, gave β,β-dimethylsuccinic acid (VI) and catalytic hydrogenation yielded β,β-dimethyladipic acid (VII). The identities of these compounds were established both by mixed melting points with synthetic products and by X-ray powder photographs. These experiments demonstrated that Simonsen's *cis*-homocaronic acid was 3,3-dimethylhexene-1,6-dioic acid (VIII). It is of interest to note that Simonsen (7) has isolated the same acid (VIII) from permanganate oxidation of Δ^3-carene and established that the two acids were identical. This is, however, quite in agreement with the findings of the preceding paragraph, since Simonsen purified the crude acids of the carene oxidation over their ethyl esters, which then were hydrolysed by strong alkali.

The present author has converted the optically active acid (IV) to optically inactive terpenylic acid (IX) by heating with concentrated hydrochloric acid in a sealed tube. Naturally, the acid (VIII) is unable to give terpenylic acid. Caronic acid is known to form terebic acid, and homoterpenylic acid is isolated after the acid isomerization of the corresponding *cyclo*propane acid, cf. Simonsen (8). Thus, the



formation of terpenylic acid is strong proof for the optically active acid (IV) to be the true homocaronic acid. Furthermore, the conversion demonstrated in the preceding paragraph gives additional proofs for the configuration of homocaronic acid, and Simonsen (7) has assumed the acid (VIII) to be a possible product of an isomerisation of homocaronic acid. Work is now under progress to synthesize homocaronic acid or its esters,[1] and until these experiments are performed it is not possible to state whether the optically active acid (IV) is the *cis-* or *trans*-modification of homocaronic acid, cf. Fredga (9).

The optical activity of the oxime (III) and the acid (IV) will very likely exclude the presence of any type of equilibrium or reactions upon oxidation as in the case of eucarvone compounds, cf. Corey and Burke (10, 11).

Reactions of carene oxime

Upon hydrolysis, an optically inactive ketone was formed, and since a seven-ring ketone, isomeric with eucarvone, was expected, the investigation was concentrated upon this compound. However, when the ketone was purified over the semicarbazone according to Widmark and Holm (12), the index-homogeneous sample gave a perfect analysis for $C_{10}H_{12}O$, viz. a loss of two hydrogens. The ketone was found to be

[1] During the preparation of this paper it was found that a purified product of the condensation of methyl 4-methylpent-3-enoate and methyl diazoethanoate in the presence of copper bronze gave an IR-spectrum which was very similar to that of the ester (V).

3,4-dimethylacetophenone (X), which was a surprising result with the mild conditions used.

Until intermediates of the conversion are isolated or established, it is impossible to give an absolute scheme of the reactions. However, a brief discussion of the conversion seems appropriate now. In the preceding chapter, carene oxime (III) is proved to contain a *cyclo*propane ring, and the position of the double bond is established also by the isolation of (+)-homocaronic acid and by the non-presence of a semi-cyclic double bond. The ketone (XI) which is related directly to the oxime will likely be converted to form an isomeric eucarvone (XII), cf. Baeyer and Wallach (13). However, no simple explanation is found for the loss of two hydrogens and migration of a methyl group, but the compound thus formed is a tropone (XIII). Now a typical product of rearrangement of this tropone would be the 3,4-dimethylacetophenone (X) isolated, cf. the review of tropones and tropolenes given by Pauson (14). It should be emphasized that the same acetophenone can be isolated from camphor which has been heated with conc. H_2SO_4 (15).

The oxime was reduced easily by sodium in ethanol to give a mixture of amines in good yield. Crystalline derivatives were also obtained in good yields, e.g. acetyl and benzoyl derivatives, picrate and hydrochloride; however, none of these compounds has yet given acceptable elementary analyses, in spite of repeated crystallizations. These compounds are now under investigation; however, in addition to isomerizations previously discussed in this paper, mixtures due to different stages of reduction, and *cis-trans*-isomerism, will not facilitate the purification.

Experimental[1]

The refractive indices are given as n_D^{25} values. The sorptograms, from the microsorption analyses according to Blohm (16), are given for this temperature and printed according to Widmark (1).

Terpene samples

The Δ^3-carene used in this investigation was of a quality corresponding to the technical sample K, cf. Widmark (1). This carene was stored for some weeks with sodium wire, then filtered and steam-distilled. For some experiments, a highly purified carene was used; sorptogram: $1.4700_{US.\ 1-6}$ 1.4703_7. The α-pinene and (+)-limonene, which have been mixed with Δ^3-carene in some experiments, were almost index-homogeneous.

Δ^3-*Carene nitrosochloride*

In order to reach the best conditions under which carene nitrosochloride (3) could be synthesized, the variations given in Table 1 were investigated. The test syntheses were performed in an apparatus containing 6 stirrers and 6 vessels which were immersed in one bath, giving the same external conditions. For every experiment, 10 ml portions of carene (or terpene mixture) were used, and the crystals were filtered after standing overnight at −20°C. The crystals were washed on the filter with cold (0°C) ethanol (95%) until all the green colour was removed. Usually, 25 ml ethanol were necessary. The samples were dried overnight at −20°C in a desiccator and then weighed. Due to the unreliability of this reaction, those syntheses were repeated in which no crystals were obtained or decomposition occurred.

[1] All melting points are uncorrected and determined in capillary tubes.

Table 1. Test syntheses of carene nitrosochloride using the amounts of reagents tabulated and 10 ml of technical Δ^3-carene.

Time for addition of HCl was 30 to 60 min. The yields of crude, but dry products are given.

No.	C_2H_5ONO ml	C_2H_5OH ml	Conc. HCl ml	HAc ml	Yield g
1	15	30	20	0	— (1)
2	„	„	„	2	1.70
3	„	„	„	5	1.80
4	„	„	„	15	1.80
5	„	„	„	20	1.65
6	„	„	„	25	1.70
7	„	10	„	10	0.80(2)
8	„	20	„	„	1.55
9	„	30	„	„	1.90
10	„	40	„	„	1.80
11	„	50	„	„	1.80
12	„	60	„	„	1.20
13	„	30	5	„	— (1)
14	„	„	10	„	1.00
15	„	„	15	„	1.30
16	„	„	25	„	1.85
17	„	„	30	„	2.00(2)
18	„	„	40	„	1.80(2)
19	6	„	20	„	— (1)
20	10	„	„	„	1.90(1)
21	20	„	„	„	1.80
22	25	„	„	„	1.40
23	30	„	„	„	0.70
24	40	„	„	„	— (1)

(1) The experiment was repeated.
(2) The crystals were difficult to filter.

The results tabulated show that the best amount of ethyl nitrite was 10 to 15 ml, and that a large excess was an obvious drawback. Presence of acetic acid was necessary, but the reaction was not sensitive to the amount used. The optimum amount of ethanol (95%) was 30 ml, but after the addition of HCl, more ethanol facilitated the precipitation. Less than 20–30 ml of conc. HCl decreased the yield, and more gave a product which was very difficult to filter. The best range of temperature was $-5°$ to $+5°$, but the danger of decomposition increased over 0°C. The time of addition of HCl did not seem to be of importance, but times under 30 min. seldom gave crystals. In every case, the rapid stirring was continued 3 hours after the addition of HCl.

In order to investigate the influence of impurities in the technical carene, syntheses were run using the pure carene mixed with pure limonene and α-pinene. Table 2 gives the yield of the crude samples. Two crystallizations of the 75% carene syntheses gave samples of carene nitrosochloride which did not depress the m.p. of a pure sample. However, there was a marked loss of material on crystallization.

Lagache (3) used 5 ml each of carene, ethanol and acetic acid, 10 ml of ethyl nitrite and 4 to 5 ml of HCl. In view of the results tabulated (Table 1), it is obvious why no precipitates were obtained in the beginning of the present investigation

Table 2. Test syntheses of terpene nitrosochlorides.

10 ml terpene, 15 ml C_2H_5ONO, 30 ml C_2H_5OH, 10 ml HAc and 20 ml conc. HCl. Pure Δ^3-carene, α-pinene and (+)-limonene were used.

Δ^3-Carene ml	α-Pinene ml	Limonene ml	Crude yield g
7.5	2.5	—	1.30
5	5	—	1.50
—	10	—	2.40
7.5	—	2.5	1.30
5	—	5	2.45
—	—	10	7.50

using the same composition and technical carene. Very pure carene gave a precipitate, but on a preparative scale even this pure carene gave an insufficient yield.

The syntheses—except for the tests given in Tables 1 and 2—were performed according to the following scheme:

150 ml carene, 150 ml glacial acetic acid, 450 ml ethanol (95%) and 225 ml ethyl nitrite were stirred vigorously in a 2-liter beaker (high model). The beaker was cooled in a bath of trichloroethylene by pieces of dry-ice. 300 ml conc. HCl were added drop-wise to the mixture which was kept at $-5°$ to $+5°$ (mostly $-5°$ to 0°). The addition took 3 to 4 hours, 200 ml of ethanol were added and the stirring was continued for a further 3 hours. The first crystals separated usually after the addition of the HCl was finished. After standing overnight at $-20°$C, the top layer and some of the thick green oil were decanted, and the crystals were filtered on a large Buchner funnel, which was surrounded by an ice bath. The crystals were sucked hard and washed with cold ethanol until all the green colour was removed. Time for suction and washing was about 4 hours. The crystals were dried on paper at room temperature for some hours and then overnight at 0°C in a vacuum desiccator. The crystals, 30 g, were dissolved in $CHCl_3$ (75 ml). The milky solution was filtered and the residue washed with $CHCl_3$ (15 ml). 90 ml of methanol were added rapidly to the $CHCl_3$ solution which precipitated crystals (25 g) of the nitrosochloride, m.p. 100–101°. These purified crystals could be stored one to two days at room temp. or about two weeks at $-20°$C.

Since the yield never exceeded 15% attempts were made to obtain more crystals from the thick green oil; this, however, failed.

Carene oxime

The conversion of the nitrosochloride to carene oxime was successful only when the reaction was performed on a small scale, cf. Lagache (3). Maximum lots of 8 g were converted but it was found most convenient to work on 4 g charges.

4 g carene nitrosochloride were suspended carefully with a few ml of ethanol to make a fine paste, which was transferred to a conical flask (500 ml). 100 to 120 ml of boiling ethanol were added and the flask was heated on a hot plate to bring most of the nitrosochloride rapidly into solution within 1 to 2 minutes. 8 ml alcoholic KOH solution (20 g KOH, 80 ml ethanol and water to make a 100 ml solution) were added, followed by 25 ml hot water after 30 sec. of heating, and again heated

shortly to give a clear, colourless solution. The solution, which must not become green or contain too much alkali, was cooled as rapidly as possible and then poured into 500 ml of ice water. After standing overnight at 0°C the crystals were filtered, washed with water and dried in a desiccator. The yield, 3 g, was dissolved in 12 ml of hot light petroleum (40–60°) and 2.5 g oxime precipitated on cooling. After two crystallizations the m.p. 97–98° was reached, and there was no further increase if the sample was crystallized from 50% ethanol. $[\alpha]_D^{25} = +374°$ (1% in abs. ethanol).

Acids from oxidation of carene oxime

8 g finely pulverized carene oxime were suspended in 400 ml of ice-water. 300 ml of permanganate solution (48.4 g $KMnO_4$ in 2 l H_2O) were added gradually at 0 to 5°C while shaking (~ 5 h). The rest of the permanganate solution was added drop by drop to the mechanically stirred suspension (0°C) during 120 hours. Excess permanganate was destroyed with 100 ml of methanol. The manganese dioxide was removed and carefully washed. The clear solution was evaporated under vacuum until about 300 ml remained in the flask. This solution was extracted 4 times with ether, acidified with H_3PO_4 and then extracted 8 times with ether. This ether solution was evaporated to give 4.4 g of a tarry oil. The oil was dissolved in dry acetone, and the addition of a slight excess of *cyclo*hexylamine in acetone precipitated the salt. The *cyclo*hexylamine salt was dissolved in a minimum amount of methanol and the salt precipitated from the clear solution by addition of acetone. After drying, these crystals melted at 196–199°, and the m.p. was not increased by further crystallizations. Analysed for N; found: N = 7.4%. (Calc. for $C_{20}H_{38}N_2O_4$: N = 7.6%.)

The acid (IV) was liberated from the *cyclo*hexylamine salt by H_3PO_4 as above, and the ether solution now gave a solid (1.5 g) upon evaporation. The acid was crystallized easily from a small amount of water, and two crystallizations brought the m.p. up to 115–117°. The specific rotation was recorded $[\alpha]_D^{25} = 62°$ (2% in abs. ethanol). Analysed for C-H; found: C = 56.0%; H = 7.3%. (Calc. for $C_8H_{12}O_4$: C = 55.8%; H = 7.3%.)

0.2 g of the acid (IV) and 2 ml acetic anhydride were heated in a sealed tube at 210° for 4 hours. After evaporation of the anhydride, the residue was distilled under vacuum (10 mm Hg), bath 160°. The drop of distillate was heated with water and upon evaporation a solid precipitated, which was recrystallized from water. This optically inactive acid melted at 133–135°, and did not depress the m.p. of an acid (VIII) prepared fully in conformity with the instructions given by Owen and Simonsen (6) for *cis*-homocaronic acid. Identical X-ray powder photographs were obtained.

0.8 g of the acid (IV) were converted to the dimethyl ester (V) by diazomethane in ether solution in the usual way. The ester (b.p. 104–106°/10 mm Hg) was optically active, $[\alpha]_D^{25} = +50°$ (2% in abs. ethanol) or $\alpha_{10\ cm} = +55.6°$ (oil) and its IR-spectrum was different from those of the dimethyl esters prepared from Owen and Simonsen's *cis*- or *trans*-homocaronic acid (VIII). The dimethyl ester (V) was analysed; found: C = 59.9%; H = 7.9%; CH_3O = 30.4%. (Calc. for $C_{10}H_{16}O_4$: C = 60.0%; H = 8.1%; CH_3O = 30.9%.) Sorptogram: $1.4461_{US,\ 1-5}$ 1.4438_6 1.4313_7. The dimethyl ester of the acid (VIII) gave the sorptogram for the *cis*-form: 1.4541_{US} 1.4539_1 1.4538_2 1.4536_3 1.4523_4 1.4419_5 1.4272_6 1.4130_7 and for the *trans*-form: 1.4460_{US} 1.4461_1 1.4433_2 1.4378_3 1.4327_4 1.4299_5 1.4291_6 1.4270_7. These three sorptograms were obtained by collecting the three first fractions from three repeated microsorptions (40 μl) of the following crude samples of the esters: (V) 1.4461_{US} 1.4462_1 1.4460_{2-3}

1.4461_{4-5} 1.4443_6 1.4360_7; (ester of *cis* VIII) 1.4535_{US} 1.4541_{1-3} 1.4504_4 1.4417_5 1.4328_6, and (ester of *trans* VIII) 1.4448_{US} 1.4460_1 1.4459_{2-3} 1.4432_4 1.4350_5.

0.3 g of the dimethyl ester (V) were warmed for 3 hours with 5 ml of a methanol solution containing 10% KOH. The methanol was then removed under vacuum and water was gradually added during the evaporation. The acid was liberated with H_3PO_4, and worked up in the usual way to give the m.p. 134–136°. This acid was found also to be identical with Owen and Simonsen's *cis*-homocaronic acid.

0.4 g of the acid (IV) were heated in a sealed tube with 3 ml of conc. HCl at 100° for 5 hours. After evaporation of the liquid, the remaining solid, after drying, was crystallized from dibutyl ether to give the m.p. 90–91°. This acid was found to be terpenylic acid (IX), which was verified by mixed m.p. and X-ray diffraction of a synthetic sample prepared according to Lawrence (17).

As reported by Owen and Simonsen (6), their *cis*-homocaronic acid was not quite stable to permanganate solution. When 0.4 g of this acid (VIII) was oxidized by permanganate solution in conformity with the oxidation given above, β,β-dimethyl succinic acid (VI) was isolated. The identity was established by the two methods previously used, and the synthetic sample was prepared according to Vogel (18).

0.1 g of the acid (VIII) was hydrogenated in acetic acid using 0.02 g of Adams' catalyst. The consumption of hydrogen was almost quantitative for one mole of hydrogen. The acid (VII) obtained was identical with β,β-dimethyladipic acid prepared according to Rydon (19), m.p. 86–87° after crystallization from dibutyl ether. The identity was established in the same manner as above.

Hydrolysis of carene oxime

10 g of carene oxime were refluxed for 2 hours with 30 g oxalic acid and 100 ml water. Steam was introduced into the flask and the oil which distilled over was separated. There was a considerable, black, tarry residue in the flask. After extraction of the distillate with ether, the ether extract was dried and evaporated to give an oil. The oil was distilled under vacuum (b.p. 110–118°/11 mm Hg) to give 1.9 g of a ketone.

This ketone sample gave an incorrect elementary analysis and showed the sorptogram: 1.5174_1 1.5178_2 1.5188_{3-5} 1.5181_6 1.5062_7. A semicarbazone was obtained easily, and the ketone was regenerated from this compound after crystallization. The ketone now gave a good analysis for $C_{10}H_{12}O$ and the sorptogram: 1.5371_{US} 1.5370_{1-6} 1.5369_7. This sorptogram was obtained also from a synthetic sample of 3,4-dimethylacetophenone (X) and there was no depression in the mixed melting points of the oximes (85–86°) or semicarbazones (232–234°).

The same ketone (X) was obtained when the hydrolysis was performed using 4 *N* H_2SO_4, and the yield was also the same. It was attempted to hydrolyse the carene oxime at room temperature by shaking a petroleum ether solution of the oxime with 9 *N* H_2SO_4. However, pure oxime was recovered from the sulphuric acid solution when this was diluted with water. This method was found useful in purifying the sticky product from the conversion of the nitrosochloride when the solution became green.

Reduction of carene oxime

In a three-necked flask, 10 g carene oxime were dissolved in 250 ml of extra dry ethanol. The solution was stirred, and slices of sodium (30 g) were added as rapidly

as possible. When the sodium had dissolved, steam was introduced into the flask to distil off the amine. The amine was separated, dried over KOH for one hour and distilled through a small column under vacuum, b.p. 76–77°/9 mm Hg. Yield 75%. Derivatives were prepared: acetyl, m.p. 142–143°; benzoyl, m.p. 150–152°, picrate, m.p. 201–203° and hydrochloride 230–231°. However, none of these compounds gave acceptable analyses for an amine $C_{10}H_{17}N$ or $C_{10}H_{19}N$.

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Studies of carane, pinane and *p*-menthane

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ABSTRACT

Δ^3-Carene, (+)-limonene, α-pinene and β-pinene have been hydrogenated to consume hydrogen corresponding to the double bonds. The products obtained have been purified to index homogeneity, carane $1.4532_{US.\ 1-7}$, pinane $1.4602_{US.\ 1-7}$, and *cis*-*p*-menthane $1.4372_{US.\ 1-7}$. Mixtures have been analysed by sorption, where good separation was reached. The same analytical result was obtained for terpenes containing small amounts of hydrogenated products.

Trans-*p*-menthane, $1.4352_{US.\ 1-7}$, prepared by electrolytic reduction of (−)-menthone, and *cis*-menthane gave no separation when mixtures of them were analysed by sorption.

In a series of earlier communications (1, 2, 3), purification of terpene hydrocarbons has been studied, using an analytical microsorption method introduced by Blohm (4). Most of the terpenes studied were unsaturated hydrocarbons, and good analytical results were obtained, However, it has become of interest for the present authors to examine hydrogenated samples of the most common constituents of Swedish turpentine. It was wished also to study the possible separation of *cis*- and *trans*-isomers.

Samples of the terpenes Δ^3-carene, α-pinene and (+)-limonene, were purified to index homogeneity, cf. Widmark (1, 2). These samples were hydrogenated at room temperature and at atmospheric pressure in an ordinary hydrogenation apparatus, using different catalysts. The obvious danger of isomerisation of the terpenes, especially that of Δ^3-carene, required that the experiments be carried out under conditions at which Δ^3-carene consumed just only one mole of hydrogen. Adams' catalyst in glacial acetic acid was the only catalyst used where reaction ceased after uptake of one mole of hydrogen, whereas for the other catalysts the consumption of hydrogen continued more or less steadily. The catalysts investigated were: platinum and palladium on charcoal in abs. ethanol, platinum and palladium-black without solvent, and Raney nickel in dioxane. Since a suitable catalyst had been found, it was of no interest for the present study to investigate further the conditions under which other catalysts would give the same results.

The solution obtained after the hydrogenation was filtered and mixed with ice-water. The hydrocarbon was separated, washed with water and $NaHCO_3$ solution, stored cold overnight with some drops of saturated $NaHCO_3$-$KMnO_4$ solution, washed again, dried over Na_2SO_4 and distilled under vacuum. The fractions from the distillation were analysed by sorption and the best fractions were purified further in lots of one to two milliliters in a large column (2 m × i. ⌀ 3 mm) but under the same conditions as for the analytical micro sorption.

The index-homogeneous samples of the hydrogenated terpenes were mixed in varying proportions and sorptograms of these mixtures were taken. In all cases,

separation was obtained, but not so distinct as in the case of the unsaturated terpenes. In an earlier paper (10), the catalytical isomerization of (−)-β-pinene to (−)-α-pinene using a palladium–$BaCO_3$ catalyst in the presence of hydrogen has been studied. The sorptograms of the α-pinene obtained usually showed a slight downward slope in the beginning of the sorptograms. The same phenomena were observed when other terpene hydrocarbons, Δ^3-carene, (+)-limonene and terpinolene were treated as above, even if they were not isomerized. The slope constituents were very likely hydrogenated terpenes, and the purification of these liquids has made it possible to study the assumed mixtures. It is now demonstrated by the sorptograms, shown in the experimental part, that there is a very good separation by the gel, and thus small amounts of hydrogenated products can be detected.

Carane, pinane and *p*-menthane all can, according to their formulae, exist as *cis-trans*-isomers. Hydrogenation of α-pinene under the conditions used is said to give the *cis* modification of pinane, whereas the *trans* form should be the result of nickel hydrogenation at 220–230°C. This last statement is somewhat questionable, cf. Lipp (5), as Ipatieff *et al.* (6) have found considerable degradation under similar conditions. Ipatieff *et al.* have found substituted *cyclo*pentanes and *cyclo*hexanes which could not very likely be removed by the simple method of purification used by Lipp. We have repeated the 220°C nickel hydrogenation, and in no case did we obtain a sample having the physical values within the range given for the *trans* isomer, or giving straight line sorptograms. However, we are conscious of the unreliability of catalysts, which naturally also makes our simple test on *trans*-pinane questionable. Moreover, pinanes having the same sorptograms were obtained independent of whether pure α-pinene or β-pinene were hydrogenated with Adams' catalyst.

In order to investigate the possible separation of *cis-trans*-isomers, a sample of *trans-p*-menthane was prepared by electrolytic reduction of *p*-menthone according to Schall and Kirst (7). Methods for the preparation of *trans*-pinane from ketones are given (8), but unfortunately we have been unable to obtain the raw materials. For these experiments with *cis-trans*-isomers we have had to be satisfied with menthane; however, we are conscious that the presence of the four-membered ring in the pinanes might have increased the possibility for separation by the gel. The samples of *trans-p*-menthane, after the usual purification, have given straight sorptograms, but we have not obtained the same level on the plateaux for different samples, in spite of using the same source of pure (−)-menthol as starting-material. Furthermore, we have not found any separation when mixtures of *cis-trans p*-menthane were analysed by sorption. Due to this observation, we cannot make any claims for the purity of our sample of *trans-p*-menthane, or any separation into *cis-trans*-isomers of the hydrogenated products investigated. We have demonstrated by experiments that our samples of the menthane isomers were stable to the silica gel and to concentrated sulphuric acid.

Experimental part

Refractometer, Bellingham & Stanley No. 397026. The instrument was calibrated against standard plates n_D 1.3922, 1.5206, 1.5240 and 1.5443. A thermostat controlled the temperature to 25 ± 0.02°C. The reproducibility of the readings was found to be ± 0.0001.

Analytical sorption apparatus. A micro apparatus (column 250 × i. ⌀ 1.4 mm),

fully observing Blohm's (4) instructions was used. The silica gel (Davison, 922-08-226 through 200 mesh) was activated for 2 hours at 140°C (15 mm Hg). Usually the gel was first dried in an oven at 100°C overnight. Abs. ethanol was used as displacer. On reading the refractive indices, a fresh paper $\sim 5 \times 5$ mm. was used for each determination. In order to simplify the printing of the sorptograms, cf. Widmark (1), and to avoid too many printed figures, the n_D^{25} values are given with the fraction number of the sorption as index. The value of the unsorbed sample is given US as index.

Sorption apparatus for purification. The column was 2 m high and had an internal diameter of 3 mm. The top of the column was blown to form a container with a capacity of ~ 30 ml. To avoid losses due to evaporation, the bottom of the column was made similar to that of the analytical column with a hole of minimum size and with a sliding set of fraction capillary pipettes. To prevent smaller particles of gel from flowing out of the hole, the bottom of the column was packed to a height of 1–2 cm with gel, 100 to 200 mesh. The rest of the column was packed carefully with the usual gel (through 200 mesh) in small portions to prevent the gel from separating when wet. After the sample had been pressed down into the top of the gel, some grains of fresh gel were added, the container was filled with abs. ethanol and connected to a cylinder of nitrogen. The pressure used to drive the sample through the column in 2 to 3 hours was usually ~ 1 kg/cm^2. There was a slight increase in temperature (max. 30–35°C) in the zone between the hydrocarbon and the displacer.

Hydrogenation apparatus. An ordinary laboratory hydrogenation apparatus of glass was used. On hydrogenation, the catalyst, the magnet covered with glass, and most of the solvent were first introduced. When the mixture had been saturated with hydrogen, the weighed terpene sample (~ 5 ml) was added air-free, and the funnel was rinsed with the same solvent. After the consumption of hydrogen had ceased, the catalyst was removed completely by filtration through very fine glass wool. Water was added carefully. The hydrocarbon layer was washed with water, then $NaHCO_3$ solution, and was stored overnight in the refrigerator with some drops of a saturated solution of $NaHCO_3$ and $KMnO_4$. After repeated washing and drying over Na_2SO_4, the hydrocarbon was distilled.

Vacuum distillations. The distillations were performed using a small, all-glass column 20 cm high, and a reflux ratio of 5:6 was maintained. In order to avoid losses due to evaporation, most distillations were carried out at 100 mm Hg.

Carane

The Δ^3-carene used for hydrogenation was purified to index homogeneity, $1.4700_{US.\ 1-7}$, and hydrogenated as above using 50 mg Adams' catalyst and 4 ml glacial acetic acid per g of terpene. 1.5 ml of the main fraction from the distillation (b.p. 90–94°C/89 mm Hg) were separated into the following fractions by sorption using the large column: 1.4487_1 1.4526_2 1.4530_3 1.4532_4 1.4533_5 1.4547_6. Fractions 3, 4 and 5 were analysed by sorption and gave the following sorptograms: Fr. 3, 1.4528_1 1.4530_{2-6}; Fr. 4, 1.4529_1 1.4530_{2-5} 1.4531_{6-7}; Fr. 5, 1.4528_1 1.4532_{2-7}. In spite of the low first figure, fraction 5 was used as the pure sample for studies of mixtures. Repeated sorption on a micro scale gave the index-homogeneous sample $1.4532_{US.\ 1-7}$. However, the optical rotations of the fractions of the large-scale sorption indicate that the purity may be doubted. In a microtube using 5% ethanolic

solutions the following specific rotations were recorded: Fr. 1, 0°; Fr. 2, −4°.0; Fr. 3, −5°.5; Fr. 4, −6°.5; Fr. 5, −9°; Fr. 6, −8°.

Hydrogenation of Δ^3-carene using palladium on charcoal showed a slow uptake of hydrogen after the consumption of one mole. In one experiment, 1.5 mole were consumed after 24 hours, and after the ordinary purification the following sorptogram was obtained, indicating obvious decomposition 1.4483_{US} 1.4390_1 1.4388_{2-3} 1.4419_4 1.4518_5 1.4604_6 1.4660_7. If the hydrogenation was stopped at one mole, a sorptogram was obtained after repeated sorptions, the middle fractions of which had the same level as the index-homogeneous carane, thus indicating the partial formation of this compound: 1.4524_{US} 1.4490_1 1.4518_2 1.4523_3 1.4529_4 1.4530_5 1.4531_6 1.4603_7. A similar condition was found for Pd-black in ethanol or acetic acid, demonstrated by the sorptogram: 1.4518_{US} 1.4491_1 1.4516_2 1.4524_3 1.4528_4 1.4530_5 1.4532_6 1.4533_7. In spite of repeated sorptions, it was not found possible to obtain samples of the same quality as in the case of the Adams' catalyst hydrogenation.

Pinane

Hydrogenation of α-pinene using Adams' catalyst gave easily an index-homogeneous sample $1.4602_{US.\ 1-7}$. In some cases, samples of this quality were obtained already after the first distillation (b.p. 90–92°C/70 mm Hg), but usually a little slope at the front and a peak at the end of the sorptogram were found, which could be removed by a 1.5 ml scale sorption. β-pinene gave the same result, but the pinane from β-pinene showed a higher specific rotation, $[\alpha]_D^{25} = -22°.5$, than that from α-pinene, $[\alpha]_D^{25} = +11°.0$. The densities in both cases were found to be $d_4^{25} = 0.851 \pm 0.002$.

In some cases the pinane obtained gave a sorptogram of slightly higher plateau but which was not quite straight 1.4606_{US} 1.4608_{1-4} 1.4606_{5-6} 1.4603_7. However, no change in specific rotation was noted; we have not found a satisfactory explanation for this result.

220°C hydrogenation of α-pinene

The hydrogenation was performed in an ordinary laboratory oven connected to a thermo couple. The catalyst, nickel prepared according to Sabatier–Senderens (13), was packed into a glass tube to a length of 30 cm. A distillation flask, filled with α-pinene was connected to the tubing. The flask was heated to 120°C, a stream of hydrogen was bubbled through the terpene, and the temperature of the oven was maintained at 220°C. After 6 hours, 200 ml of distillate had condensed in a cooling bath at the other side of the oven. The product was distilled carefully through a column under vacuum to give 8 fractions. The fractions 5 and 6 (b.p. 94–98°C/87 mm Hg) having the n_D^{25} values 1.4672 and 1.4691 were investigated further. The first fractions were, according to their sorptograms, mostly α-pinene. 20 ml of the fractions mentioned were treated with small portions of a saturated solution of $NaHCO_3$ and $KMnO_4$. A considerable reaction took place. 120 ml were added over a period of several days and the temperature was kept at 2–5°C. Excess permanganate was destroyed by methanol and the mixture was extracted with ether. After washing etc., 2.6 g liquid were recovered, and distilled in one fraction giving an inexplicable sorptogram: 1.4650_{US} 1.4627_1 1.4652_2 1.4661_3 1.4669_4 1.4674_5 1.4442_6.

p-Menthane

The refractive indices of the four fractions on vacuum distillation of a hydrogenated sample of (+)-limonene were found to be: Fr. 1, 1.4371; Fr. 2 and 3, 1.4373; Fr. 4,

1.4378 (b.p. 51.5–52.5°C/10 mm Hg). A large scale sorption of fraction 3 (1.5 ml) gave the following 6 fractions: Fr. 1, 1.4378; Fr. 2–5, 1.4372; Fr. 6, 1.4369. Sorption of fraction 4 gave an index-homogeneous sample $1.4372_{US.\ 1-7}$. The first and the last figures changed rapidly upon storing and gave then 1.4378 and 1.4370, respectively. This sample was used as the pure sample of *cis-p*-menthane for the mixtures. There is a notable difference in the refractive indices (calculated for 25°C) given by O'Connor and Goldblatt (11), 1.4411, and by Keats (12), 1.4391. As in case of pinane, a higher plateau was sometimes observed, represented by the sorptogram: 1.4375_{US} 1.4379_1 1.4378_{2-4} 1.4377_5 1.4376_6 1.4373_7.

Some samples of *trans-p*-menthane were prepared according to Schall and Kirst (7). The cadmium electrode (2 mm thick plate, 1 × 1 dm) was bent to fit into an 800 ml beaker. The lead electrode, a spiral (area slightly smaller than the Cd electrode), was arranged in a porcelain cylinder. Both electrodes were made from A.R. grade metals. The electrolysis of menthone and the first stages of purification were performed according to the instructions given. The menthone, prepared from (−)-menthol (14), m.p. 32°, was not found to be index-homogeneous, and gave the sorptogram: 1.4489_{US} 1.4487_{1-2} 1.4488_3 1.4489_4 1.4491_5 1.4492_6.

On distillation of menthane (b.p. 96–98°C/87 mm Hg) three fractions were obtained: Fr. 1, 1.4350; Fr. 2, 1.4352; Fr. 3, 1.4353. Fraction 2 gave the sorptogram 1.4353_1 1.4352_{2-7} and after purification by a large scale sorption, the index-homogeneous sample $1.4352_{US.\ 1-7}$ was found. However, one hydrogenation experiment gave a menthane, which upon purification gave an index-homogeneous sample $1.4368_{US.\ 1-7}$. This was first interpreted as being due to a partial isomerization by the gel to the *cis* form.

In order to investigate the danger of isomerization, two tests were made with *cis-* and *trans*-menthane. Small samples were stored 2½ days with a few drops of conc. H_2SO_4 at room temperature, and two 80 μl samples were sealed in tubes with silica gel and heated at 100°C overnight. The *cis*-menthane changed slightly, showing different values for only the first and last figure of the sorptogram as in the case of ordinary storing. There was no change at all for the *trans*-menthane which gave again the sorptogram $1.4352_{US.\ 1-7}$. Thus, these two experiments demonstrate that the lift of the plateau mentioned cannot be due to acid isomerization.

Sorptograms of mixtures of carane, pinane and p-menthane

The mixtures were prepared with the aid of small capillary pipettes, usually in 50 to 70 μl quantities. The accurancy of the composition of the mixture was of the order of 5 per cent.

Carane–pinane

28 % carane
1.4602_{1-4} 1.4592_5 1.4569_6 1.4552_7

51 % carane
1.4602_1 1.4601_2 1.4584_3 1.4563_4 1.4551_5 1.4542_6 1.4539_7

75 % carane
1.4582_1 1.4561_2 1.4548_3 1.4543_4 1.4540_5 1.4539_6 1.4400_7

*Carane–*cis-p-*menthane*

35 % carane
1.4378_1 1.4372_{2-3} 1.4382_4 1.4411_5 1.4469_6 1.4510_7

49 % carane
1.4377_1 1.4372_2 1.4389_3 1.4439_4 1.4482_5 1.4510_6 1.4522_7

76 % carane
1.4399_1 1.4461_2 1.4492_3 1.4510_4 1.4520_5 1.4528_6 1.4530_7

Pinane–cis-p-*menthane*

52 % pinane
1.4531_1 1.4510_2 1.4497_3 1.4489_4 1.4479_5 1.4472_6 1.4461_7

75 % pinane
1.4598_1 1.4579_2 1.4562_3 1.4546_4 1.4530_5 1.4512_6 1.4492_7

90 % pinane
1.4602_{1-3} 1.4598_4 1.4587_5 1.4568_6 1.4542_7

cis-p-*Menthane*–trans-p-*menthane*

23 % cis-p-menthane
1.4358_{1-7}

54 % cis-p-menthane
1.4363_{1-7}

77 % cis-p-menthane
1.4370_1 1.4368_{2-7}

Terpenes mixed with small amounts of hydrogenated terpenes—sorptograms

5 % *cis-p*-Menthane in (+)-limonene
1.4588_1 1.4698_2 1.4702_3 1.4703_{4-7}

11 % Pinane in β-pinene
1.4678_1 1.4732_2 1.4750_3 1.4754_4 1.4758_5 1.4761_6 1.4762_7

11 % Pinane in α-pinene
1.4623_1 1.4628_2 1.4630_3 1.4631_4 1.4632_{5-7}

10 % Carane in Δ^3-carene
1.4610_1 1.4681_2 1.4692_3 1.4698_4 1.4699_5 1.4700_{6-7}

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Autoxidation of (+)-limonene

By Gunnar Widmark

With 2 figures in the text

ABSTRACT

(+)-Limonene, purified to index homogeneity, has been oxidized to a consumption of 5 and 80 mole per cent of oxygen, respectively. No decrease in the rotation of the recovered limonene, which has been isolated by extraction and purified to index homogeneity, was observed.

The author has been unable to isolate an optically inactive hydroperoxide from the oxygenated mixture, but index-homogeneous (±)-carvone has been obtained after sulphite reduction and chromate oxidation, even from the 5 per cent autoxidation. To avoid the loss of small amounts of optically active carvone, no crystallisation methods have been used throughout the scheme of purifications.

The experiments performed support the assumption of a radical mechanism for the autoxidation of (+)-limonene.

Blumann and Zeitschel (1) in 1914 claimed to have isolated optically inactive carvone from a sample of (+)-limonene, which they had had under autoxidation for months. Recently, the isolation of optically active carvone (2) has been discussed. The rotation of the carvone obtained from (+)-limonene is, as will be demonstrated later, a matter of theoretical interest. Since the present author (3) had earlier purified and analysed samples of (+)-limonene the problem was open for study.

Index-homogeneous (3) (+)-limonene, $1.4703_{US.\ 1-7}$, was oxidized by oxygen at room temperature in diffuse daylight. An ordinary hydrogenation apparatus was used. The oxidation was usually continued to a consumption of 0.8 mole of oxygen, but in order to study the phase of initiation, some oxidations were stopped when 0.05 mole had been consumed by the (+)-limonene. The oxygen-containing compounds produced by the oxidation were removed by extraction using 90% methanol, cf. Widmark (4). The methanol solution was extracted by light petroleum to remove remaining small quantities of limonene. Most of the methanol was evaporated using a water pump at 20 mm Hg (bath temp. max. 20°), the residue was treated with ether to remove water, and after drying (Na_2SO_4) the ether was evaporated, finally at 0.5 mm Hg. These samples will be referred to in the following as raw or crude limonene hydroperoxide.

Some samples of the raw hydroperoxide from the low degree of oxidation were distilled under high vacuum (10^{-4}) at room temperature. A small fore-fraction, a large middle-fraction and a residue were obtained, all being optically active. The activity was not changed after standing for a fortnight at room temperature. The middle-fraction gave a perfect oxygen analysis for $C_{10}H_{16}O_2$, but the iodine titrations gave, as usual, unreliable results, cf. Blohm and Widmark (5). When this purified

sample was reduced by neutral sodium sulphite solution, a product was obtained, which after distillation under vacuum gave a good analytical result for a terpene alcohol $C_{10}H_{16}O$. Even this purified sample was optically active.

Bluhmann and Zeitschel have shown this alcohol to contain (±)-carveol from the mixed melting point determination of the phenylurethane. Carveol was later separated into *cis-trans*-isomers (6). The present author has verified the presence of (±)-carveol by isolating the 3,5-dinitrobenzoate of (±)-*trans*-carveol and proved this by comparison with a synthetic sample.[1] However, in both these cases, small amounts of optically active carveol could have been lost by the repeated crystallisations. In order to make sure that no optical activity was present, the reduced hydroperoxide was oxidized by chromate solution, which gave a sample of carvone after sulphite extraction (1). This sample of carvone had a very low rotation, and was not pure when analysed by sorption. Girard-P extraction gave, however, an index-homogeneous carvone, which was totally inactive optically. Natural (+)-carvone has a high rotation, $\alpha_{10\,cm} = +57.1°$, and this means that it would have been possible to detect less than half a per cent optically active carvone by this experiment. Furthermore, it has been shown that there is no loss in activity when (+)-carveol is oxidized by chromate and the (+)-carvone obtained is purified to index homogeneity by these two extractions. Experiments to isolate other ketones from the chromate oxidized sample of reduced hydroperoxide have been unsuccessful up to now, as also attempts to separate the alcohol 3,5-dinitrobenzoates by chromatography.

The residual sample of raw limonene hydroperoxide from the higher degree (0.8 mole) of autoxidation was extracted directly after the reduction by neutral sulphite solution and gave a considerable amount of carvone, which was identified by its semicarbazone and 2,4-dinitrophenylhydrazone, verified by mixed melting point determinations of derivatives of a known sample of (±)-carvone. Further extraction by Girard-P reagent gave, as before, an index-homogeneous sample of carvone, for which no rotation could be detected. Fresh samples of the low degree of oxidation (0.05 mole oxygen) gave no yield of carvone on sulphite extraction. However, samples of the low-oxidized limonene, which were stored airtight for one month before the isolation of the hydroperoxide gave a very small yield of (±)-carvone upon extraction. I have not yet succeeded in isolating crystals of limonene glycol, but I have not had the opportunity to store the sample as long as, e.g., Schmidt (7) has reported.

The first reason to oxidize limonene to the high consumption of oxygen, was to study the optical properties of the limonene which was recovered in the residue after the methanol extraction. Repeated distillations under vacuum gave a sample of limonene, the sorptogram of which showed a straight line with a peak at the end. The "peak" was removed by running a large sorption purification (tube 2 m × i. ∅ 3 mm) using the same gel and the same conditions as for the sorption analysis. The index-homogeneous sample of limonene obtained was examined in the polarimeter. The same rotation value was found as for the purified starting material (4).

Discussion

It is not the intention of the present author to bring new physical evidence for the complex scheme of autoxidation, but to discuss how the experimental facts fit in

[1] The residual mother liquids from the crystallisation of the 3,5-dinitrobensoate have been too complex to allow isolation of the *cis*-isomer.

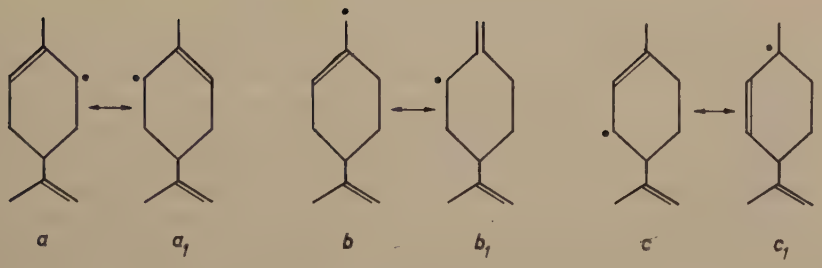

Fig. 1. Assumed radicals of limonene.

with the theory given by the British school of Farmer *et al.* (8), and reviewed by Bolland (9) and Bateman (10). This school considers the autoxidation to proceed by a three-stage radical mechanism: initiation, propagation and termination. During the first two stages the formation of hydroperoxides from radicals dominates, and in the last the radicals are transferred to non-chain-reacting compounds. The active centres of the molecule are considered to be not the carbon atoms of the double bond, but the methylene groups on each side of the double bond. Bolland (11) has given the relative reactivity of, e.g., the olefinic group $R\text{–}\overset{a}{C}H_2\text{–}C(CH_3)=CH\text{–}\overset{c}{C}H_2\text{–}R$ to be 1:3:11, and the reactivity is influenced by the nature of the substituents CH_2R (10). According to Bolland, the exocyclic double bond of limonene must give rise to a slower rate of oxidation than the cyclic one; therefore the compounds derived from the former are left out of discussion, at least for the low degree of oxidation (cf. below).

In agreement with the theory reviewed, the three active methylene groups of limonene, *a*, *b* and *c*, give the corresponding radicals demonstrated in fig. 1 by the structures *a*, *b* and *c* and their resonating structures a_1, b_1 and c_1.

The conformity of consumption of oxygen with that liberated by the iodine titration in the first part of the autoxidation of limonene is an indication that a monohydroperoxide is formed (see Fig. 2). Furthermore, the same slope of the percental decrease in optical rotation of the sample is noted. Unfortunately, the method of determination (titration) of the hydroperoxide on a concentrated sample is unreliable, but the oxygen analysis is in good agreement with that of the hydroperoxide $C_{10}H_{16}O_2$. Therefore, the formation of a hydroperoxide is assumed.

The limonene hydroperoxides derived from the four structures *b*, b_1, *c* and c_1 will all theoretically give optically active hydroperoxides, whereas the radical *a* is symmetrical by resonance and will give an optically *inactive* hydroperoxide. The optical activity of the sample from the 0.05 mole oxidation is taken to mean that the sample is a mixture of the hydroperoxides.[1] Unfortunately, it has not yet been found possible to separate this assumed mixture by a 25 tube counter-current apparatus, or by chromatography. Dimerisation may make separation impossible. According to Bolland (11), the optically active peroxides of methylene group *c* should dominate the hydroperoxide mixture (cf. verbenone from oxidized α-pinene), but the high symmetry of radical *a* must influence the composition towards the inactive hydro-

[1] The isolation of an optically inactive dimer of the radical *a* would be an excellent proof for the real formation of the radical. However, I have not succeeded in detecting this compound.

peroxide. A quantitative determination of the content of (±)-carveol is planned using infrared spectroscopy.

By experiments the sensitivity of which is better than half a per cent, it is shown that the conversion of carveol to carvone and the purification of carvone does not involve losses or conversion of optically active material. Thus, assumed mechanisms for the autoxidation involving the formation of optically active products from these two compounds must be excluded in all the three periods of the reaction mentioned above. However, the present author has not found it possible to demonstrate experimentally that the reduction of hydroperoxide to alcohol is not accompanied by conversion. This means that the hydroperoxides derived from some of the optically active radicals, e.g. c_1, may be inactivated by the reduction. However, the conformity of the rates of oxygen consumption and decrease in activity in the first part of the autoxidation is an argument against this. The structure *c* would give an optically active alcohol which, however, would very likely be converted by the chromate oxidation to an optically inactive ketone, cf. piperitenone–isopiperitenone. Furthermore, I have found that chromate oxidation of the alcohols from reduction of Δ^3-carene hydroperoxide gives only a very small yield of a ketone mixture.

Of the endocyclic peroxides, the one bridging the carbon atoms 2 and 6 would give a symmetrical molecule resembling carveol, and Khan (12) has assumed a mechanism of reaction, demonstrated on olive oil, which may be able to produce this 2,6-peroxide. It is not very likely that this peroxide would be reduced to an alcohol, cf. ascaridol. A more obvious explanation for the inactivation discussed is a reaction involving the hydrogen of the asymmetrical carbon atom 4, e.g., due to the effect of the exocyclic double bond of limonene. A hydroperoxide at carbon atom 4 would not explain the formation of (±)-carveol, except for some inactivation of limonene itself, before or on reaction of atoms 2 and 6. The unchanged rotation of the (+)-limonene recovered from the 0.8 mole autoxidation contradicts, however, this assumption. Furthermore, the assumption of a recombination of limonene from the radical *a* is disqualified by this observation.

Simonsen (13) assumes the glycol of limonene to be an intermediate in the formation of (±)-carveol and (±)-carvone. Schmidt (7) has isolated the glycol from a very old sample of oxidized limonene and established its optical activity. Since the radical mechanism allows the formation of the optically active glycol from a reaction of (+)-limonene and the decomposing hydroperoxide, the assumption mentioned is not longer probable.

Experimental part

The microsorption analyses have been performed according to the instructions given by Blohm (14). All the determinations of refractive indices have been made at 25°C and all the sorptograms are given for this temperature. For further details concerning the use of the microsorption method, the system to report the sorptograms and the purification of the terpenes by displacement chromatography, cf. e.g. Holm and Widmark (15).

(+)-Limonene (Eastman-Kodak T 1980) was prepared easily in large quantities in a state of index homogeneity, $1.4703_{\text{US. 1–7}}$, cf. Widmark (4). The apparatus for the oxidations was one formerly used for hydrogenation, and was placed near a window in the laboratory. The capacities of the containers for oxygen and water were ~ 1.5 l. Usually one mole of limonene was placed in a conical flask (1 l) which

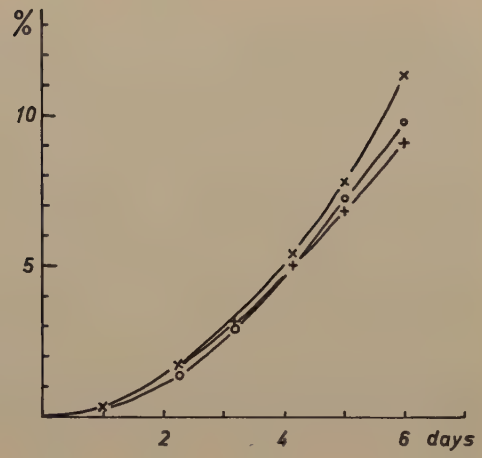

Fig. 2. Autoxidation of (+)-limonene. ×, Mole per cent oxygen consumed. ○, Mole per cent hydroperoxide from iodine titrations. +, Percental decrease in optical activity.

was connected to the oxygen container. The oxidation was run at a slight pressure (~25 cm water) and took 3 to 5 days for the 0.05 mole oxidation and 14 to 20 days for the 0.8 mole oxidation, depending more on the temperature than on the illumination. The consumption of oxygen was recorded daily, and for some experiments the changes in peroxide content (iodine titration) and optical rotation were determined. Approximate conformity with the experiments on a smaller scale reported by Widmark and Blohm (16) was found, and the three quantities mentioned followed each other closely up to five mole per cent oxidation. (See Fig. 1.)

The products formed by the oxidation were separated from the hydrocarbon by five to six methanol extractions. 25 ml portions of methanol, which contained 10% water, were used. The combined methanol solutions were extracted with light petroleum until the petroleum phase was free from optical rotation. This was valid for the low oxidations but the 0.8 mole oxidations were extracted just four times. The residual methanol solution was evaporated at room temperature using a water pump. A thick oil mixed with water was finally formed. The oil was dissolved in ether. The ether solution was dried (Na_2SO_4) and evaporated, finally at 0.5 mm Hg. Attempts were made to determine the peroxide content by titration, but both the iodine method and the method of Barnard and Hargrave (17) failed because duplicate tests varied usually by 10% or more.[1] This is notable since both tertiary butyl peroxide and pinane hydroperoxide gave constant analyses.

0.05 mole oxidation

The original intention of this experiment was to isolate a pure sample of an optically ainctive limonene hydroperoxide. Since the crude sample of the peroxide had a rotation $[\alpha]_D^{25} = +41°$ attempts were made to purify this sample. High vacuum distillation (10^{-4} mm Hg) at room temperature gave a large middle fraction, $[\alpha]_D^{25} = 38°$, a small first fraction $[\alpha]_D^{25} = 39°$ and a residue $[\alpha]_D^{25} = 40°$. All rotations were determined

[1] The titrations showed hydroperoxide content to vary between 85 % and 110 % for different samples prepared in the same way, but variations of the same order were obtained with each of the two methods used.

using absolute ethanol as solvent. The middle fraction was analysed for oxygen by combustion, found O 18.67%. Calc. for $C_{10}H_{16}O_2$: O 19.03%. Counter current extraction in a set of 25 tubes gave no separation and the same negative result was found using chromatography on activated aluminium oxide.

The crude peroxide was reduced by neutral sulphite solution. 10 g peroxide were dissolved in 10 ml of methanol and added to a solution of 15 g Na_2SO_3 and 2 g $NaHCO_3$ in 150 ml of water. The suspension was stirred vigorously, cooled by tap water in the beginning and then heated to 40°C. After 4 hours, the mixture was cooled and poured into 200 ml of water. The oil was separated, and the water solution was extracted 2 times with 100 ml lots of ether. The combined oil and ether solution was washed several times with water, dried with Na_2SO_4, evaporated and distilled through a laboratory column, cf. Table 1. It was not found possible to treat these fractions of terpene alcohols by the analytical sorption method. This was demonstrated by the sorptogram given for a sample of (+)-*trans*-carveol liberated from a carefully purified 3,5-dinitrobenzoate. 1.4947_{US} 1.4942_1 1.4930_2 1.4896_3 1.4824_4 1.4730_5 1.4510_6. The sorptograms of the fractions 3 and 7 were: 1.4857_{US} 1.4867_1 1.4859_2 1.4848_3 1.4770_4 1.4650_5 and 1.4933_{US} 1.4893_1 1.4772_2 1.4712_3 1.4630_4 respectively. Similar sorptograms, difficult to interpret, were obtained from most terpene alcohols investigated.

Fractions 3, 6 and 9 of the distillate (Table 1) were treated with 3,5-dinitrobensoyl chloride and pyridine in the usual way. After ten to fifteen crystallisations, samples of correct melting point for (±)-*trans*-carveol 3,5 dinitrobensoate, m.p. 119–120.5°C, were found which did not depress the m.p. of a synthetic sample prepared according to Johnston and Read (6). However, due to the repeated crystallisations the loss of optically active material was obviously possible. A redistilled sample of the combined fractions 3, 6 and 9 was analysed for C-H, found: C 78.9 %, H 10.5 %. Calc. for $C_{10}H_{16}O$: C 79.0 %, H 10.6 %.

The combined fractions of the distillate were oxidized by potassium dichromate solution and the tarry oil obtained was extracted with neutral sulphite solution, cf.

Table 1. Distillation of reduced limonene hydroperoxide in an all-glass laboratory column (60 cm high).

Reflux ratio 12:13. Time 5.30 h.

Fraction No.	Weight g	Temp. °C	Temp. of bath °C	Press. mm Hg	$[\alpha]_D^{25}$ in ethanol	n_D^{25}
1	2.45	83	133	9.9	117.0	1.4729
2	2.60	97	136	10.2	82.5	1.4831
3	2.77	97	138	10.2	85.0	1.4857
4	2.80	99	140	9.6	85.8	1.4869
5	3.09	101	141	9.6	80.0	1.4880
6	2.91	102	141	9.6	76.5	1.4891
7	2.91	106.5	143	9.6	6.9	1.4993
8	2.93	108.5	145	9.6	13.9	1.4931
9	2.50	110.2	145	9.6	27.5	1.4922
10	2.64	112.0	146	9.6	41.8	1.4912
11	3.26	113.2	146	9.6	62.5	1.4903
12	2.86	—	146	9.6	72.0	1.4933
Residue	3.30					1.4948

Bluhmann and Zeitschel (1). The ketone mixture liberated was distilled under vacuum and the alcoholic residue was oxidized again. The ketone gave the sorptogram: 1.4963_{US} 1.4968_1 1.4969_{2-5} 1.4964_6 1.4908_7; $\alpha_{10\ cm} = 4.7°$ (oil in micro tube). This sample of carvone was purified to index homogeneity $1.4969_{US,\ 1-7}$ by two methods, Girard-P extraction and displacement chromatography (column 2 m high, i. ∅ 3 mm), cf. Widmark and Holm (18). The index-homogeneous sample gave a zero rotation (0.03°) when investigated in a microtube (5 cm). The yield of the chromate oxidation was only 10% and a similar yield was obtained when 4 g pure carveol (from $LiAlH_4$ reduction of (+)-carvone) were oxidized and gave 0.45 g ketone. This last carvone sample, after purification by a large-scale sorption, gave an index-homogeneous sample, which had the same rotation, $\alpha_{5\ cm} = 28.6°$, as a highly purified sample of (+)-carvone (18): $\alpha_{10\ cm} = +57.1°$.

Sulphite extraction of freshly prepared, reduced limonene hydroperoxide gave no yield of carvone, but an oxidized sample, 10 g, which was allowed to stand in the refrigerator for one month gave a very small sample of carvone, less than 20 mg, just sufficient to prepare a semicarbazone for determination of melting point and mixed melting point.

0.8 Mole oxidation

After the consumption of 0.8 mole oxygen, the terpene sample was usually slightly yellow and turbid. The methanol and light petroleum extractions were performed as before. The crude sample of peroxide $[\alpha]_D^{25} = 35°$ (2% in absolute ethanol) had to be reduced first by sulphite before the extraction of the carvone using the same reagent. The yield of crude carvone liberated from the sulphite extract and the water phase of the reduction was 2.4 g from 16 g of crude peroxide. This sample gave a sorptogram similar to the one from the low-degree oxidation. An index-homogeneous sample of (±)-carvone was obtained after the purification as used before. The 2,4-dinitrophenylhydrazone of (±)-carvone and the semicarbazone, m.p. 187–188°C and 154–156°C, respectively, were easily obtained and found to be identical with synthetic samples by mixed melting points.

The residue of impure hydrocarbon terpene after the methanol extraction was 38 g from the oxidation of one mole (+)-limonene. The middle fraction (17 g) from the distillation of this sample was impure according to the sorptogram: 1.4750_{US} 1.4699_1 1.4702_2 1.4703_{3-4} 1.4748_5 1.4808_6 1.4832_7. Repeated distillation under vacuum gave a more pure sample, 10 g, sorptogram: 1.4707_{US} 1.4703_{1-5} 1.4706_6 1.4718_7. 1.5 ml of this sample was run through the large gel column, which gave some fractions (0.6 ml) of $n_D^{25} = 1.4703$ in conformity with the micro sorptogram. A new analytical sorptogram established the index homogeneity of the limonene recovered, $1.4703_{US,\ 1-7}$. Combined samples of this quality gave the same rotation as that of the index-homogeneous starting material, $\alpha_{10\ cm} = 103.3°$. Index-homogeneous (+)-limonene of the same rotation was recovered also by repeated extractions with methanol (90%).

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Zur Kinetik der Reduktone und Pseudo-Reduktone I

Von Hans von Euler and Hans Hasselquist

Mit 6 Figuren im Text

Die Kinetik der Reduktone berührt zwei grosse und wesentliche Forschungs-Aufgaben, nämlich A: eine physikalisch-chemische — die Wirkung der Reduktone als Funktion atomarer Eigenschaften und der Struktur ihrer Moleküle zu erkennen, und B: eine biochemische — die Vorgänge zu ermitteln, durch welche die Reduktone als hormonale Komponenten der Zellen in den tierischen und pflanzlichen Stoffwechsel eingreifen.

A. Der zeitliche Verlauf der Reaktion der Reduktone mit Oxydationsmitteln sowie mit Sauerstoff wurde zuerst von Euler und Martius (1) sowie von Euler, Myrbäck und Larsson (2) untersucht und in diesem Institut dann zur Charakterisierung verschiedener Klassen von Reduktonen verwendet. Besonders eingehend wurde hierbei die Wechselwirkung verschiedener Klassen von Reduktonen mit Tillmans Reagens (TR, Dichlorphenol-indophenol) unter verschiedenen Reaktionsbedingungen (Acidität, Lösungsmittel) studiert. Bald hatte sich gezeigt, dass 1. die Geschwindigkeit der untersuchten Reaktion stark von der Alkalinität der Lösung (pH) abhängt (1, 3), und dass 2. bei aci-Reduktonen[1] — z. B. Triose-Redukton, Ascorbinsäure — die Kurven, welche die Reaktionszeiten des TR mit pH in Beziehung setzen, ein Minimum der Geschwindigkeit, also ein Maximum der Reaktionszeiten aufweisen (4).

Zu berücksichtigen ist hierbei, wie schon 1934 von Martius und Euler betont wurde, dass auch die zweite Reaktionskomponente, TR, in ihrer Aktivität von pH beeinflusst wird. Wie aus der Fig. 1 hervorgeht, lässt sich im pH-Gebiet 2–13 spektrometrisch *keine* Strukturänderung nachweisen. Damit ist allerdings eine Aktivitätsänderung des TR im erwähnten pH-Bereich nicht ausgeschlossen.[2]

Das aci-Redukton Ascorbinsäure

Ascorbinsäure und TR. Die pH-Abhängigkeit der Ascorbinsäure ist in diesem Institut wiederholt untersucht worden. Dabei hat sich stets der schon von Euler, Myrbäck und Larsson (2) beobachtete Einfluss des Phosphat-Puffers auf Höhe und Verlauf der pH-Kurve ergeben, der auch bei unseren neuen Messungen zum Ausdruck kommt (Fig. 2 *b*).

[1] Zur Nomenklatur der Reduktone siehe Euler, Kem. Arb. N.F. BI, Bd 16, III (1954).

[2] Über das oxydo-reduktive Verhalten des Leuko-Dichlorphenol-indophenols siehe Euler u. Hasselquist, Die Reduktone, Stuttgart, Verlag Enke (1950), S. 40.

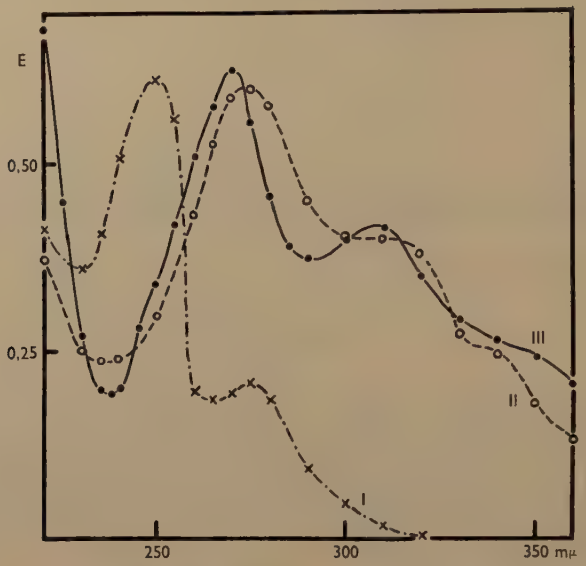


Fig. 1. Spektrum von Dichlorphenol-indophenol (TR). I. Anfangs rot, dann farblos; pH = 2. II. Umschlagspunkt, rot-blau; pH = 5,5. III. pH = 6–13.

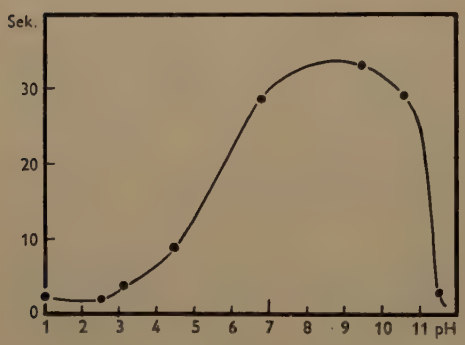


Fig. 2 *a*. Ascorbinsäure, ohne Puffer, 1,0 ml 0,005-mol., 0,8 ml 0,01 TR.

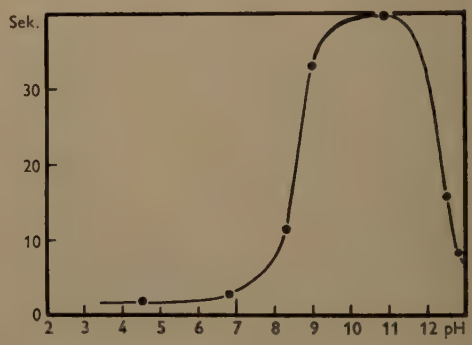


Fig. 2 *b*. Ascorbinsäure, Phosphatpuffer, 1 ml 0,005-mol., 0,8 ml 0,01-mol. TR, 0,2 ml $^1/_{15}$-mol. Phosphatpuffer.

Bei den Versuchen von EULER, HASSELQUIST und CEDER (3) erwies sich die Reaktionsgeschwindigkeit wenig aber deutlich abhängig von der Konzentration der Ascorbinsäure, die zwischen n/5 und n/250 variiert wurde. Nach den Versuchen von CEDER (3) liegt das Minimum der Reaktionsgeschwindigkeit in der Pufferlösung bei pH 11, in der pufferfreien Lösung um rund 2 pH-Einheiten niedriger.

Unsere neuen Versuche (Fig. 2 *a* und 2 *b*) zeigen wieder die grosse Reaktionsgeschwindigkeit in alkalischer und — besonders auffallend — auch in stark saurer Lösung. An letzterer Erscheinung ist vielleicht das TR beteiligt.

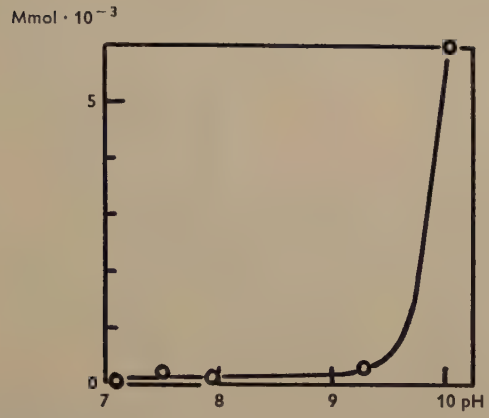
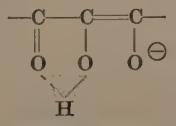


Fig. 3. 8 ml 0.005-mol. Ascorbins.-Lös. + 2 ml NaOH-Lös. + O_2 zurücktitriert mit 0,005-mol. TR-Lös.

Ascorbinsäure und J_2. Wie KARRER und Mitarb (5) nachwiesen, verbraucht Ascorbinsäure in saurer Lösung 2 Atome Jod; sie geht dabei in Dehydroascorbinsäure über (Arkiv Kemi *4*, 170, 1951/52). In alkalischer Lösung fanden sowohl KARRER als wir bei Überschuss von Jod und dem anderthalbfachen der berechneten Menge NaOH in 15 Min. 4 Atome Jod per Mol Ascorbinsäure.

Zum Vergleich haben wir auch die Geschwindigkeit gemessen, mit der Ascorbinsäure durch molekularen Sauerstoff oxydiert wird. Wir liessen hierbei reines Sauerstoffgas durch Ascorbinsäure-Lösungen perlen[1]. Die Ascorbinsäure wurde in den von Zeit zu Zeit entnommenen Proben titrimetrisch bestimmt. Die Fig. 3 zeigt, dass ohne Zusatz von Metallkatalysatoren erst über pH 9 eine schnellere Oxydation der Ascorbinsäure eintritt. Bei Zusatz von Cu^{++} tritt die früher von MYRBÄCK und LARSSON (2) gemessene Metallkatalyse auf, wodurch die Kurve der Fig. 3 bei pH 6,5 ein Maximum erhält.

Was nun die reaktionsvermittelnde Molekülart in alkalischen Ascorbinsäure-Lösungen betrifft, so beginnt von etwa pH 4–5 an das mono-valente Ascorbinsäure-Anion, das vielleicht teilweise in Chelatform vorliegt, aufzutreten. Wie aus der Titrationskurve, Fig. 3, hervorgeht, macht sich von pH 9 an auch das divalente Ascorbinsäure-Anion geltend.



Chelat-Form

Über die Form der Ascorbinsäure, welche in mässig saurer Lösung (pH ~ 1–4) die Oxydo-Reduktion mit TR vermittelt, liegen noch keine entscheidenden Daten vor. Ascorbinsäure-Kationen, die in Betracht zu ziehen sind, konnten noch nicht sicher nachgewiesen werden.

[1] Auch bei diesen Versuchen sowie im System Ascorbinsäure, Sauerstoff Methylenblau tritt die Puffer-Wirkung hervor (EULER, MYRBÄCK, LARSSON, l. c. Seite 8). Bez. der Oxydation der Ascorbinsäure und des Triose-Reduktons durch Sauerstoff siehe EULER u. HASSELQUIST (Z. physiol. Chem. 288, 4 u. zw. 9 (1951).

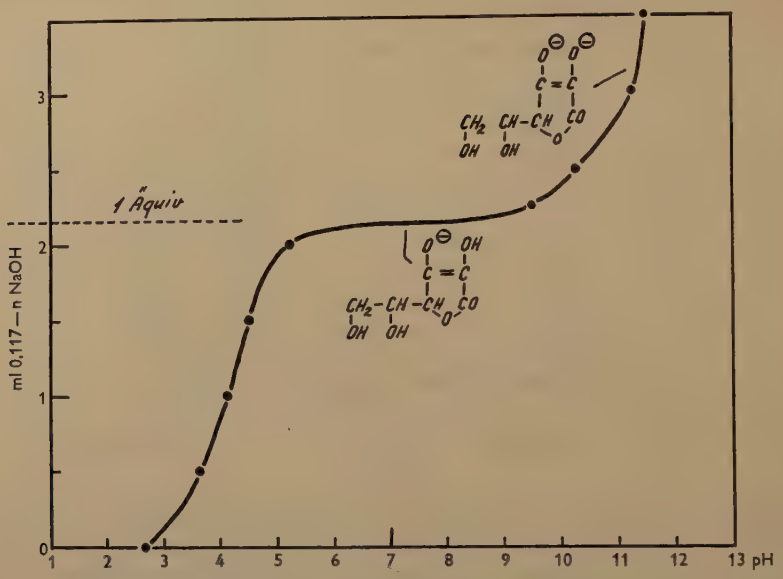


Fig. 4. 2,5 ml 0,1-mol. Ascorbinsäure. Titrierungskurve.

Vergleicht man die Geschwindigkeitskurve der Oxykomensäure mit den Kurven des Ascorbinsäure, so findet man das Minimum der Geschwindigkeit bei annähernd dem gleichen pH. In neutraler Lösung (pH 7) sind die Reaktionszeiten (ohne Puffer): für Ascorbinsäure 33 Sek., für Oxykomensäure 150 Sek.

Eine ähnliche Abhängigkeit der Reaktionsgeschwindigkeit mit TR zeigt sich ausser bei Triose-Reduktone auch u. s. bei der *Dioxy-maleinsäure* (Lieb. Ann. Chem. 581, Kurve S. 207) und bei der Oxykomensäure (6).

Pseudo-Reduktone

Reduktone, deren Endiol-Gruppe nicht einem Carbonyl (oder einem anderen sauren Rest) benachbart ist, sind nur als *Reduktonate*, u. zw. durch ihre Anionen, wirksam. Die Geschwindigkeit ihrer Reaktion mit TR nimmt von pH 7 an mit steigendem pH zu, z. B. beim Pyrocatechol (Kem. Arb. N. F. B, 18, II 1956.

Bei den *aci-Reduktonen* liegt das Keto-Enol-Gleichgewicht auch in saurem Medium so weit auf der Enol-Seite, dass eine rasche TR-Reaktion eintreten kann, wie dies bei der Ascorbinsäure der Fall ist.

Nun kennt man neben den durch ihren Endiol-Gehalt charakterisierten, stark reduzierenden Reduktonen noch eine grosse Klasse von Substanzen, welche, unter gewissen Bedingungen, *ähnlich wie Reduktone reagieren, ohne die Endiol-Gruppe zu enthalten.* Wir bezeichnen dieselben als „*Pseudo-Reduktone*". Damit sollen die Stoffe zusammengefasst werden, die keine Reduktone sind, aber sich z. T. wie solche verhalten und Reduktone entstehen lassen können. In dieser Hinsicht wäre vielleicht die Bezeichnung „Pro-Reduktone" zutreffend[1].

[1] Wir zählen zu den Pseudo-Reduktonen also nicht solche Stoffe wie Hydrazine, welche ebenfalls TR oder andere Test-Substanzen der Reduktone reduzieren.

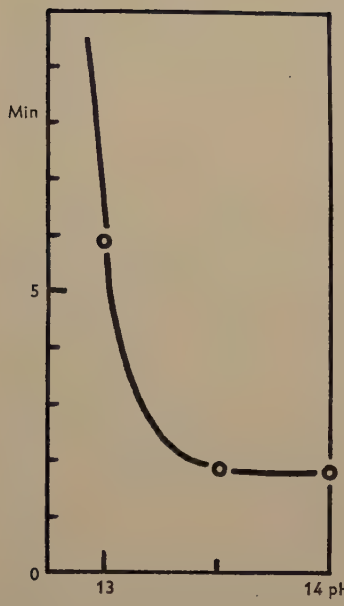


Fig. 5. 1 ml 0,005-mol. Diacetbernstein-säureester 0,8 ml 0,005-mol. TR.

Indessen ist die Nomenklatur-Frage hier nicht die Hauptsache. Im Wesentlichen handelt es sich darum, den Zusammenhang zwischen der in biochemischer Hinsicht bemerkenswerten Klasse der Reduktone und einer noch umfassenderen Klasse von Substanzen klarzustellen und die Mechanismen und Gesetzmässigkeiten ihrer Übergänge aufzuklären. (Auf eine rationelle Unterteilung in die recht verschiedenen Arten von Pseudo-Reduktonen sowie auf ihre Keto-Enol-Gleichgewichte nach K. H. MEYER, DIECKMANN, ARNDT-MARTIUS, EISTERT und BRIEGLEB kommen wir in einer folgenden Mitteilung zurück.)

Als Beispiele erwähnen wir zunächst Vertreter der *Diketone*, deren Enole bereits beschrieben sind[1].

Diacetbernsteinsäure-ester

Das aus Acetessigester und Jod in bekannter Weise dargestellte aus Alkohol umkristallisierte Präparat schmolz bei 90°. Lösten wir diese stabile Form des Esters in Wasser auf und titrierten nach Alkalisierung mit 2 n. NaOH, so wurden in langsamer Reaktion 85% des Esters oxydiert. Wie Fig. 5 zeigt, setzt die Reaktion mit TR erst in stark alkalischer Lösung, über pH 12, ein.

Jod wird in neutraler Lösung nicht verbraucht. Liesen wir den Ester in alkalischer Lösung mit Jod 30 Min. stehen, säuerten dann an und titrierten mit Thiosulfat zurück, so betrug der Jodverbrauch von 1 Mol Ester 1,1 Mole J_2.

[1] Übergänge von Diketonen und Diketosäuren in ihre Enole haben wir hier einstweilen nur studiert, soweit sie in *wässriger* Lösung eintreten. Wir dehnen unsere Untersuchung auf Enolisierungen in anderen Lösungsmitteln aus, besonders in Rücksicht auf die interessanten Ergebnisse von HENECKA sowie besonders von BRIEGLEB und seiner Schule.

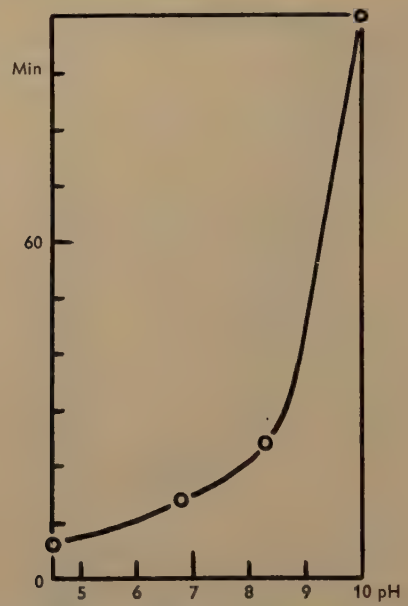


Fig. 6. 1 ml 0,005-mol. Dihydroxyfuran-dicarbonsäureester. 0,8 ml 0,005-mol. TR. 0,2 ml Phosphatpufferlösung.

Sehr langsame Übergänge in Formen, die mit TR in Reaktion treten, findet man u. a. bei folgenden Diketosäuren und Diketonen:

α,α'-*Diketo-adipinsäure-ester* wurde nach der von KUHN und DURY (7) angegebenen Methode durch Bromierung von Brenztraubensäure-äthylester im Licht dargestellt. Wie bekannt geht die α,α'-Diketo-adipinsäure auch in saurer Lösung in Dihydroxy-muconsäure über.

Benzoyl-formoin (ABENIUS u. SÖDERBAUM, Ber. 24 u. 25; 1891/92). Lässt sich mit TR in saurer Lösung in 20–30 Sekunden titrieren. *E* gef. 120, *E* ber. für $C_{16}H_{12}O_4$ 134. Jod-Verbrauch durch Endiolat von KARRER (Helv. Chim. Acta 18) festgestellt.

Succinylo-bernsteinsäure-ester (H. LIEBERMANN, Ann. 404, 1914). Verbraucht per Mol in alkalischer Lösung 0,97 Mol. TR.

Unter den Diketonen, die unserer Feststellung zufolge weder in saurer noch netraler oder alkalischer Lösung reduzieren, seien erwähnt:

Das 1,3-Diketon *Acetylaceton*. Ferner *Acetonylaceton* (verbraucht aber Jod), *Oxalyl-di-acetophenon* (C. BEYER u. L. CLAISEN, Ber. 1887), *Phenacetyl-acetophenon*, 2-*Benzoyl-cyclopentanon*, *Oxal-diessigsäureester*, 1,2,3-*Tribenzoyl-cyclopropan*.

Die Pseudo-Reduktone sind aber keineswegs auf lineare oder zyklische Diketone (*Vinylogen* nach KLAGES (8) beschränkt, deren Aktivität man in verschiedener Weise beschreiben bzw. klarstellen kann, sei es unter Hinweis auf die Resonanz zwischen den beiden Carbonylgruppen, sei es auf Grund des Vinylogen-

Prinzips von ANGELI und KLAGES, oder im Sinne der Vakanz-Theorie von THIELE. Die Substanzen, die wir in dieser vorläufigen Übersicht hier noch nennen wollen, haben uns schon als Vorstufen von Bis-Reduktonen in chemischer und in biochemischer Hinsicht interessiert.

Vorstufen von Bis-Reduktonen

Das *Bis-Redukton aus 3,4-Dihydroxy-furan-2,5-dicarbonsäure-ester* reagiert mit TR wesentlich langsamer als z. B. das aci-Redukton Ascorbinsäure bei gleicher Acidität. Die in Fig. 6 angegebenen Reaktionszeiten sind wesentlich höher als die für die Ascorbinsäure (Fig 2 *a*) oder für Triose-Redukton gefunden; die Reaktionszeiten sind wohl im wesentlichen bedingt durch die Geschwindigkeit der Ringspaltung des Furanringes bzw. durch die Geschwindigkeit der Bildung des entsprechenden Bisreduktones, das in die Enolform übergeht.

Dem obenerwähnten Dihydroxy-furan-dicarbonsäure-ester entspricht der *N-Phenyl-3,4-dihydroxy-pyrrol-2,3-dicarbonsäure-ester*. Dieser Ester und die anologe phenyl-freie Verbindung unterscheiden sich auffallend hinsichtlich ihres Verhaltens zu TR. Die N-Phenyl-Verbindung wird durch Alkali unter Bildung eines Bis-endiols leicht aufgespalten, während dies beim phenylfreien Ester nicht der Fall ist (9). Nach halbstündiger Einwirkung von 2-norm. Alkali tritt nämlich keine mit einer Ringöffnung verbundene zweite Endiolbildung ein, während in saurer Lösung (pH ~ 4) sofort 1 Mol TR unter Bildung einer Endiol-Gruppe reduziert wird.

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Investigation of carvone and related ketones

By Gunnar Widmark and Beryl Holm

ABSTRACT

Carvone has been isolated from spearmint oil, caraway oil and autoxidized (+)-limonene. Independent of direction of rotation and the methods used here for purification, the final products all showed the index-homogeneous sorptogram $1.4969_{US,\ 1-7}$.

Index-homogeneous carvone has been converted into carone, carvenone, carvotanacetone, dihydrocarvone and eucarvone. These ketones have been purified to index homogeneity; carone: $1.4758_{US,\ 1-5}$ 1.4759_{6-7}, carvenone: $1.4808_{US,\ 1-7}$, carvotanacetone: $1.4782_{US,\ 1-7}$ and eucarvone: $1.5070_{US,\ 1-7}$. Dihydrocarvone has been split up by sorption into two plateaux, $1.4682_{US,\ 1-7}$ and $1.4700_{US,\ 1-6}$ 1.4702_{7}, respectively, but definitive proofs for them to be the *trans-* and *cis-*modifications are lacking. The sequence of the ketones (50 % mixtures), viz. the fall in affinity for the gel, was found to be: carvenone, carvone, carone, carvotanacetone, dihydrocarvones, eucarvone.

Sorptograms from mixtures of these ketones show good separations, except for carone, which means that the index-homogeneous result for carone has to be treated with care. The effect of storing has been studied briefly.

Ketones which are isomeric with, or derivatives of, carvone are described frequently in the chemical literature, cf. Simonsen (1). The interest in these compounds is not only a matter of relative accessibility but they are, as most terpenes, suitable for theoretical studies. However, a necessary condition for such studies is the use of pure, unmixed compounds. The present authors, who are studying the compounds formed by the autoxidation of terpenes, describe in this paper the purification and micro-sorption analysis of some of the ketones in this group.

Carvone possesses a central position in this investigation since it is an intermediate in the preparation of all the other ketones investigated. Therefore, the purification of carvone has been studied most thoroughly. Samples of carvone were isolated from different sources, spearmint oil, caraway oil and (+)-limonene. In the last case, carvone was obtained both optically inactive from an autoxidized sample and in an active form by chemical reaction of the nitrosochloride in the usual way over the oxime. A number of different methods to isolate and purify the samples were used, in order to check the analytical result. We have found the combination of sulphite extraction and purification over the semicarbazone to give the best results.

At every stage of the purification, whenever a liquid sample of carvone was present, we have analysed the sample using the micro sorption method of Blohm (2), and finally obtained samples having the same sorptogram $1.4969_{US,\ 1-7}$ for (+)-, (−)- and (±)-carvone, and similar values of rotation for the antipodes. We consider this

observation to be a good proof for the presence of a pure sample of carvone, but we wish here to call attention to the danger in claiming purity from only a single straight sorptogram, cf. carvone, Widmark and Blohm (3), and mixtures of carone, see below. The pure carvone obtained was used for analytical studies and to prepare the other ketones of this investigation.

Dihydrocarvone

Carvone was reduced by zinc according to Wallach (4). Samples of dihydrocarvone purified over the semicarbazone gave sorptograms having two plateaux, the first being well defined and the latter more diffuse and of a higher refractive index. This behaviour was changed very little in favour of the lower plateau when the semicarbazone was crystallized repeatedly, the ketone purified over the sodium hydrogen sulphite complex, or carefully fractionated under vacuum.

We have performed the sorption using the 2 m column and 1.5 ml samples, and reached a separation similar to that of the micro sorption analysis. 40 μl samples of the two plateaux were analysed by sorption showing index homogeneity for the lower plateau and an almost straight line for the higher plateau. Both gave a good C-H analysis for $C_{10}H_{16}O$. If samples of the two plateaux were converted to semicarbazones, crystallized and then liberated, sorptograms similar to those of the original dihydrocarvone were obtained. Samples of the higher plateau showed higher optical rotation than those of the lower plateau, and because good sorption separation was given by the other ketones—except for carone—these must be excluded as participators in a possible mixture.

This behaviour may be taken as an indication of separation into *cis*- and *trans*-isomers, where, according to what is usually accepted, the lower plateau is the *trans*-dihydrocarvone and the higher the *cis*-isomer. We had not expected a separation of the isomers by the gel since our attempts to separate mixtures of the isomers of *p*-menthane had been unsuccessful, cf. Holm and Widmark (5). However, it is possible that the double bond of dihydrocarvone facilitates the separation.

Carvotanacetone

We have prepared carvotanacetone by partial hydrogenation of carvone using Adams' catalyst, and by bromination of carvomenthone according to Kötz and Steinhorst (6). In spite of the opinion of Simonsen ((1), I, 341), the reduction was found to be by far the best method for the preparation of pure carvotanacetone. The ketone liberated from the semicarbazone by phthalic anhydride gave no index-homogeneous sample, but samples cut out of large sorptions were found to be index-homogeneous $1.4782_{\text{US. }1-7}$ independent of direction of rotation. The best fractions from the large sorption of samples prepared by the method of Kötz and Steinhorst had to be purified over the semicarbazone once more to give straight sorptograms of the same value as before.

Carvenone

We have prepared this ketone by careful isomerization of dihydrocarvone using cold concentrated sulphuric acid according to Baeyer (7). The purification was performed over the semicarbazone. The semicarbazone was found to be more difficult

to purify by crystallization than the others, and only samples with melting points reaching 200°C were fit for purification. Semicarbazones of a slightly lower melting point were found usually to decrease in melting point on recrystallization. Even the free carvenone was found to be unstable when sorbed through the large column. A pure sample, $1.4808_{US.\ 1-7}$, which gave a semicarbazone of melting point 201°C was changed to $n_D^{25} = 1.4822$ when sorbed through a large column. This sample gave a semicarbazone melting at 198–199° and, after three crystallizations, 196–199°.

Eucarvone

The instructions given by Baeyer (8) and Hershberg (9) were used for the preparation of eucarvone and the ketone was purified over the semicarbazone. The semicarbazone was easily purified and converted to the ketone, but the eucarvone was somewhat difficult to obtain in an absolutely index-homogeneous form. The deviations were very small and only in one case we have found a quite straight sorptogram $1.5070_{US.\ 1-7}$. We have no definite proofs for isomerizations caused by the gel, and there was very little difference between the refractive indices of the fractions of the large sorption and the micro sorption.

Carone

We have prepared our carone by the method given by Klotz (10), and have used the unsorbed, but semicarbazone-purified, dihydrocarvone as starting material. Since carone could not be regenerated unchanged from the semicarbazone, cf. Gillam and West (11), this method has been excluded. Instead, we have attempted the purification of the dihydrocarvone hydrochloride by sorption, which has not been successful, probably on account of unfavourable viscosity. Carefully fractionated dihydrocarvone hydrochloride has been used for the alkaline conversion to carone, and the product obtained was fractionated through a column under vacuum. One of the fractions was found to be index-homogeneous and this fraction has been used as pure carone for the studies of sorption analysis of mixtures.

Carone should exist in *cis-* and *trans*-isomers but, judging from the behaviour of the *p*-menthanes sorption (5), separation was not likely to occur here. Thus, an inseparable mixture of *cis-trans*-isomers was assumed to be present. Furthermore, as seen from the sorption studies of mixtures, carone mixtures were usually very little separated when sorbed through the gel column. This means that we cannot guarantee the absence of small amounts of the other ketones in our sample of carone, but small amounts of carone in mixtures of the other ketones could be removed by crystallization of the semicarbazones.

Sorption studies of ketone mixtures

These analyses have been performed to give additional proof of the purity of our ketones which have been purified to index homogeneity. As seen from the sorptograms given in the experimental part, good separation by the gel was obtained in all cases except for carone mixtures. All the possible pairs of mixtures, using the ketones given, have not been investigated, but enough were made to determine the sequence, viz. the falling affinity for the gel, given for 50% mixtures, which is found to be: carvenone, carvone, carone, carvotanacetone, dihydrocarvone, eucarvone and

menthone.[1] The mixtures were prepared using calibrated micro pipettes. Smaller deviations of the index homogeneity of the ketones for the mixtures were tolerated since it was found difficult to have enough freshly prepared ketones at the same time.

In connection with the study of mixtures, properties of some ketones were investigated briefly and illustrated by sorptograms. Sorptograms demonstrating the effect of storing, oxidation, interaction of gel and height of the analytical column are given at the end of the experimental part. No definite conclusions can be drawn from these sorptograms, but there was no marked isomerization caused by the gel on prolonged heating. However, the change caused by storing and autoxidation was notable. This last observation, which is in no way original, demonstrates the importance of using freshly prepared samples of terpene compounds for physical studies.

Experimental part[2]

Refractometer, Bellingham & Stanley, No. 397026. The instrument was calibrated against standard plates n_D 1.3922, 1.5206, 1.5240 and 1.5443. A thermostat controlled the temperature to 25 ± 0.02°C. The reproducibility of the readings was found to be ± 0.0001.

Analytical sorption apparatus. A micro apparatus (column 250 × i. ⌀ 1.4 mm), fully observing Blohm's (2) instructions, was used. The silica gel (Davison, 922-08-226 through 200 mesh) was activated 2 hours at 140°C (15 mm Hg). Absolute ethanol was used as displacer. On reading the refractive indices, a fresh paper ~ 5 × 5 mm, was used for each determination. In order to simplify the printing of the sorptograms, cf. Widmark (12, 13), and to avoid too many printed figures, the n_D^{25} values are given with the fraction number of the sorption as index. The value of the unsorbed samples is given US as index.

Sorption apparatus for purification and distillation apparatus were the same as used in a previous work, but the output of the distillations were usually smaller, cf. Holm and Widmark (5).

Purification of carvone

The raw materials for the preparation of the enantiomorphs of carvone were the two technical essential oils, caraway oil and spearmint oil; sorptograms: 1.4852_{US} 1.4728_{1-2} 1.4818_3 1.4897_4 1.4921_5 1.4931_6 1.4840_7 and 1.4863_{US} 1.4757_1 1.4832_2 1.4901_3 1.4910_4 1.4900_5 1.4878_6 1.4823_7 respectively.[3] These two oils were distilled through a small all-glass column under vacuum without giving any pure fractions according to the sorptograms, which is demonstrated by the sorptograms of the fractions from the distillation of spearmint oil (Table 1). Better results were obtained when the oils were extracted first by neutral sulphite solution, the water phase separated, treated with excess sodium carbonate, and steam-distilled to liberate the ketones (cf. Bluhmann and Zeitschel (14)), Tables 2 and 3.

The yield from the extraction was not quantitative, and some decomposition of carvone was caused also by the sulphite, which was demonstrated by extraction of a pure (see below) sample of carvone. 3 g lots of carvone were shaken 1, 2, 4 and 6

[1] Menthone was prepared according to *Organic Syntheses* II, 240, and the middle fraction gave upon distillation under vacuum the sorptogram 1.4480_{US} 1.4482_1 1.4480_{2-5} 1.4482_6 1.4422_7.

[2] All melting points are uncorrected and determined in capillary tubes.

[3] These oils were obtained from Standard Synthetics Ltd., Barnes, London.

Table 1. Distillation of spearmint oil in an all-glass laboratory column at 15 mm Hg. Reflux ratio 9:10.

Fraction No.	Temp. °C	Refractive indices of sorptograms, n_D^{25} 1. ...
1	61–67	4672_{US} 4670_1 4691_2 4700_3 4708_4 4718_5 4706_6 4718_7
2	67–106	4710_{US} 4711_1 4719_2 4728_3 4690_4 4687_5 4703_6 4430_7
3	106–108	4902_{US} 4862_1 4878_{2-3} 4882_4 4891_5 4912_6 4783_7
4	109	4932_{US} 4908_1 4922_{2-3} 4918_4 4922_5 4940_6 4730_7
5	110	4942_{US} 4931_1 4938_{2-5} 4940_6 4847_7
6	110.5	4945_{US} 4940_1 4950_{2-4} 4942_5 4939_6 4832_7
Residue		5068_{US}

Table 2. Distillation of (+)-carvone extracted by sulphite solution from caraway oil.

Column 20 cm high. Pressure 14 mm Hg. Distillation temperature 112–112.5°C. Reflux ratio 14:15.

Fraction No.	Refractive indices of sorptograms, n_D^{25} 1. ...
1	4949_{US} 4943_1 4949_2 4951_{3-4} 4952_{5-6} 4948_7
2	4952_{US} 4951_1 4952_2 4953_{3-6} 4937_7
3	4956_{US} 4953_1 4958_{2-6} 4580_7
4	4959_{US} 4959_{1-5} 4955_6 4798_7
5	4959_{US} 4960_{1-5} 4958_6 4882_7
6	4959_{US} 4960_{1-2} 4961_{3-5} 4958_6 4954_7
Residue	4962_{US}

Table 3. Distillation of (−)-carvone extracted by sulphite solution from spearmint oil.

Conditions: see Table 2.

Fraction No.	Refractive indices of sorptograms, n_D^{25} 1. ...
1	4950_{US} 4948_1 4949_2 4950_3 4944_4 4941_5 4942_6 4840_7
2	4969_{US} 4959_1 4960_{2-5} 4959_6 4981_7
3	4969_{US} 4961_1 4962_{2-6} 4959_7
4	4969_{US} 4965_1 4962_{2-4} 4963_5 4968_6 4941_7
5	4968_{US} 4962_1 4963_{2-5} 4860_6 4332_7
6	4962_{US} 4963_{1-6} 4939_7
Residue	4969_{US}

hours with 40 ml sulphite solution in a four-armed shaker (Microid, Griffin & Tatlock Ltd., London). After careful treatment with ether, 0.5, 0.29, 0.28 and 0.21 g, respectively, of unextracted material was recovered. Repeated extraction of the combined residues, 1.28 g, gave a new residue, 0.65 g, which after distillation under vacuum gave a sorptogram 1.4893_{US} 1.4901_1 1.4906_{2-3} 1.4908_4 1.4904_5 1.4762_6. 6.8 g of pure, unchanged carvone was recovered from the combined sulphite solutions.

The ketone liberated from the sulphite extract was converted easily to the crystalline semicarbazone in the usual way, but the yield never exceeded 80%. The unextracted essential oils gave only slowly crystalline samples of semicarbazone and in poor yield. The samples of semicarbazone were crystallized from ethanol to give the two modifications with the melting points 161–162° and 140–141° respectively, but only one for the optically inactive form 154–155°. We have not found any regularity of melting point and method of crystallisation; cf. Rupe and Dorschky (15) who have found only the higher melting modification of (−)-carvone semicarbazone. Melting-point determinations of mixtures of equal quantities of the enantiomorphs always had m.p. 154–155°, whereas other mixtures gave the long range melting points 140–160°, which often was found for samples after crystallization.

Due to this irregularity in the melting points of the semicarbazones, we have crystallized the samples only once before regenerating the carvone. One part semicarbazone was mixed thoroughly with one and a half parts phthalic anhydride and the mixture steam distilled. The ketone layer thus liberated and distilled was separated and dried over sodium sulphate. Sometimes the water layer was extracted, and we have found light petroleum to be superior to ether in this connection. Upon vacuum distillation, samples of carvone were obtained which were analysed by sorption. The sorptograms were of the shape represented by the example 1.4968_{US} 1.4767_{1-2} 1.4769_{3-6} 1.4766_7, but in some few cases index-homogeneous samples were obtained, $1.4969_{US.\ 1-7}$. Steam distillations using half-saturated oxalic acid gave very similar results, but we preferred phthalic anhydride which gave a quicker distillation. In every case it was found necessary on vacuum distillation to remove a first fraction of low refractive index, e.g. 1.4950_{US} 1.4961_1 1.4965_{2-3} 1.4966_{4-5} 1.4888_6 1.4553_7.

To obtain index homogeneity of the not quite pure samples regenerated from the semicarbazones, two methods were found suitable, extraction by Girard-P reagent, and displacement chromatography. Two grams of ketone, 50 ml abs. ethanol, 6.7 g Girard-P reagent and 6.7 ml glacial HAc were refluxed for one hour. The mixture was cooled and poured into 300 ml ice water containing 5.6 g Na_2CO_3. The solution was extracted three times with ether (evaporation gave only traces of an oil $n_D^{25} = 1.5409$) and cooled again. Then 11 ml conc. HCl were added cautiously and the solution allowed to stand one hour at room temperature, when four extractions with ether were made. The ether extract was washed, dried, evaporated and distilled under vacuum. The yield was 75 per cent of index-homogeneous carvone. A sample of this quality was boiled for 2 hours with conc. HAc. Unchanged carvone was recovered.

Displacement chromatography using the same gel as for the micro sorption analysis also gave an index-homogeneous carvone. We have used a tube (2 m high, i. ∅ 3 mm), cf. Holm and Widmark (5), and displaced 1.5 ml samples through gel (Davison 922 × 08 × 226) by abs. ethanol under pressure. Seven 0.2 ml fractions were usually taken of which refractive indices were recorded, exemplified by: Fr. 1, 1.4963; Fr. 2, 1.4968; Fr. 3–6, 1.4969; Fr. 7, 1.4971. Combined fractions 3 to 6 gave an index-homogeneous micro sorptogram $1.4969_{US.\ 1-7}$ and in the case of optically active material $\alpha_{10\ cm} = +57.1°$ and $-57.8°$.

The preparative sorptions were performed also using larger samples through tubes of greater internal diameter. Similar results were obtained for i. $\varnothing = 4$ mm and 2 ml samples, but i. $\varnothing = 4.8$ mm and 3 ml samples gave non-index-homogeneous middle fractions when they were analysed by micro sorption.

The semicarbazones were also converted to a ketonic sample by steam-distillation over 4 *N* H_2SO_4. This method, which was used in the beginning, has caused us much trouble, since varying results were obtained. In some cases we have found samples having sorptograms under the 1.4969 level, e.g., 1.4939_{US} 1.4933_1 1.4935_2 1.4940_{3-6}, but mostly higher sorptograms were recorded, e.g., 1.5158_{US} 1.5159_1 1.5161_2 1.5152_3 1.5022_4 1.4920_5 1.4774_6, and in one case the misleading result $1.4959_{US.\ 1-6}$ was obtained. Furthermore, these samples gave semicarbazones both slowly and in insufficient yields. Girard extraction gave a sample of lower sorption level, 1.4960_{US} 1.4962_{1-7}, but repeated crystallisations of the semicarbazone of the Girard extract gave finally, when the phthalic anhydride method was used, an index-homogeneous sample $1.4969_{US.\ 1-7}$. The optical rotations were lower, e.g. $\alpha_{10\ cm} = 22.4°$ for the 1.5158_{US} sample given above. Unfortunately, some commercial samples of carvone had apparently been regenerated by the sulphuric acid method.

Carvone formed readily a crystalline adduct with hydrogen sulphide. These crystals usually separated more easily from an impure carvone mixture than did the semicarbazone. However, the yield was much lower, only $\sim 25\%$ from pure carvone. Carvone was regenerated from the pure sulphide by KOH both in water, methanol and ethanol, and after vacuum distillation samples having the same index homogeneity were obtained $1.4969_{US.\ 1-7}$, cf. Wallach (16).

Limonene forms optically inactive carvone on autoxidation. One of us (17) has reported the isolation of an index-homogeneous, $1.4969_{US.\ 1-7}$, sample of carvone which was found to be totally inactive optically. (+)-Limonene can be converted to (−)-carvone via the nitrosochloride and the oxime. We have prepared a sample of oxime, which was steam distilled together with oxalic acid to give an impure carvone, sorptogram: 1.4950_{US} 1.4930_1 1.4952_2 1.4960_3 1.4965_4 1.4968_5 1.4970_6. When purified over the semicarbazone in the usual way, an index-homogeneous (−)-carvone $1.4969_{US.\ 1-7}$ was obtained.

Dihydrocarvone

Dihydrocarvone was prepared according to Wallach (4) by zinc reduction of a semicarbazone-purified sample of carvone. After distillation (115–120°C, 15 mm Hg) to remove the pinacone, the distillate, sorptogram 1.4698_{US} 1.4347_1 1.4632_2 1.4661_3 1.4670_4 1.4678_5 1.4682_6 1.4696_7, was converted in good yield, $\sim 90\%$, to the semicarbazone, which after one crystallization was converted to the ketone by the phthalic anhydride method, yield 90%. The dihydrocarvone was distilled under vacuum to give four similar fractions; sorptogram of fraction 2: 1.4687_{US} 1.4682_{1-3} 1.4685_4 1.4688_5 1.4689_6 1.4690_7 1.4691_8. Distillation through a column raised the first plateau slightly 1.4683_{US} 1.4682_{1-5} 1.4687_6 1.4688_7. Repeated crystallization of the semicarbazone before the dihydrocarvone was liberated gave a sorptogram very similar to the latter one given above.

The raw dihydrocarvone, after the first distillation, gave readily a crystalline sodium hydrogen sulphite complex in a yield of $\sim 40\%$. Dihydrocarvone was regenerated by 10% NaOH, washed, dried and distilled under vacuum to give a main fraction, sorptogram $1.4682_{US.\ 1-2}$ 1.4683_{3-6} 1.4689_7, viz. an increase of the lower level.

On displacement chromatography of 2 ml through the large column (2 m × i. ⌀ 3 mm), 1.2 ml had the n_D^{25} value of 1.4682 and 0.5 ml had n_D^{25} values between 1.4692 and 1.4700; a small middle fraction was discarded. The two fractions were re-sorbed. The lower plateau then had the same level and the index-homogeneous sorptogram, $1.4682_{US,\ 1-7}$. The higher plateau gave sorptograms, slightly varying e.g. 1.4698_{US} 1.4696_1 1.4697_{2-3} 1.4699_{4-5} 1.4670_6 1.4671_7 and in the best case $1.4700_{US,\ 1-6}$ 1.4702_7. The spec. rotations $[\alpha]_D^{25} = \pm 12°$ and $\pm 15°$ (2% in abs. ethanol) were found for the low and the high level, respectively, and elementary analyses gave: C = 78.5%, H = 10.8% and C = 78.7%, and H = 10.8%, respectively. (Calculated for $C_{10}H_{16}O$: C = 78.8%, H = 10.6%.)

Samples of the two plateaux were converted to semicarbazones, which were crystallized, both having the same melting point 181–182.5°C and the same mixed melting point; however, they gave different spec. rotations; the lower level semicarbazone $[\alpha]_D^{25} = -17°$ and the higher $-70°$ (1% in abs. ethanol). Dihydrocarvone regenerated from these two semicarbazones gave similar sorptograms resembling rather more the sorptograms of the samples from which they were derived: 1.4683_{US} 1.4682_{1-5} 1.4685_6 1.4692_7 and 1.4686_{US} 1.4682_{1-2} 1.4688_3 1.4690_4 1.4694_5 1.4697_6 1.4695_7, respectively.

We have prepared the crystalline oximes and 2,4-dinitrophenylhydrazones of the two plateaux, both pairs having the same melting points, 90–91° and 158–159°, and the same mixed melting points. Only in one case of a sample from the micro sorption have we had a pair of 2,4-dinitrophenylhydrazones melting at 146–147° for the lower plateau and 125.5–126.5° for the higher; mixed melting point 102.5–106°. Girard-P extraction gave only 40% yield and no further separation of the plateaux.

Carvotanacetone

Pure samples of carvone (3 g) were hydrogenated using Adams' catalyst (80 mg) in glacial acetic acid (10 ml). The reaction was stopped when one mole of hydrogen had been consumed because there was no change in rate of consumption of hydrogen at this point, cf. Vavon (18). The raw ketones were converted to semicarbazones and crystallized until the m.p. 171–172°C (opt. act. material) was reached. The ketone was regenerated using phthalic anhydride in the ordinary way to give 0.6 g raw material. Sorptogram: 1.4772_{US} 1.4770_1 1.4772_2 1.4778_3 1.4779_4 1.4781_5 1.4784_6 1.4788_7. Samples (1.5 ml) of similar qualities were sorbed through the large column (2 m × i. ⌀ 3 mm) to give 0.6 ml of a middle fraction $n_D^{25} = 1.4782$, which gave an index-homogeneous sorptogram $1.4782_{US,\ 1-7}$. Occasionally, a very slight lift of the plateau to 1.4784 was observed, independent of whether (+) or (−) carvone was used as starting material.

Carvotanacetone was also prepared using the method of Kötz and Steinhorst (6). Carvone was hydrogenated to carvomenthone having the sorptogram 1.4521_{US} 1.4520_{1-3} 1.4521_{4-5} 1.4522_6. 20 g carvomenthone gave 5 g crude carvotanacetone, which was distilled under vacuum to give the following sorptogram of the best fraction: 1.4720_{US} 1.4718_{1-3} 1.4720_4 1.4719_5 1.4697_6. This fraction was converted to the semicarbazone, m.p. after recrystallizations 168–170°C. The ketone was liberated (0.46 g) to give the sorptogram 1.4772_{US} 1.4780_1 1.4778_2 1.4774_3 1.4773_{4-5} 1.4778_{6-7}. The fraction from a large sorption (n_D^{25} 1.4774) gave, after purification over the semicarbazone, a small amount of index-homogeneous material.

Carvenone

The instructions given by Baeyer (7) were followed to prepare carvenone from dihydrocarvone and cold conc. H_2SO_4. The purification was performed over the semicarbazone, m.p. after recrystallization 199.5–200.5°. We have not found the lower-melting modification. A 4 g sample of dihydrocarvone gave 1.8 g carvenone, regenerated from the semicarbazone. 0.8 g index-homogeneous carvenone $1.4808_{US.\ 1-7}$ was obtained on distillation under vacuum. In order to obtain more material of this quality, a large sorption was performed, which, however, gave a middle fraction with micro sorptogram 1.4822_{US} 1.4830_1 1.4822_{2-5} 1.4823_6 1.4808_7. This sorbed sample gave a semicarbazone 198–199°, which had a lower m.p. after crystallization. Since the large sorption was unsuccessful as a method of purification, samples varying slightly from the 1.4808-level were used to prepare mixtures.

Eucarvone

24 g Carvone were converted to eucarvone according to Baeyer (8), using abs. HBr in glacial acetic acid, to give 21.5 g of crude eucarvone. Distillation under vacuum gave a main fraction, sorptogram: 1.5050_{US} 1.5049_1 1.5058_2 1.5055_3 1.5054_4 1.5050_5 1.5047_6 1.5040_7. After two crystallizations the semicarbazone melted at 184–187°C. The ketone was liberated as usual to give on vacuum distillation a middle fraction with sorptogram $1.5069_{US.\ 1}$ 1.5070_{2-6} 1.5068_7. Combination of the fractions 2–6 from two micro sorptions gave a new sorptogram $1.5070_{US.\ 1-7}$, viz. index homogeneity. Similar sorptograms were obtained when the ketone was liberated by pyruvic acid, cf. Hershberg (9). The 1.5070 sample from a large sorption gave micro sorptograms slightly varying, e.g. 1.5068_1 1.5070_2 1.5071_{3-6} 1.5060_7 and 1.5070_{1-6} 1.4942_7.

Carone

Carone was prepared from (+)- and (−)-carvone according to Klotz (10). Since Gillam and West (11) have shown the purification over the semicarbazone to give decomposition of carone, we attempted to purify dihydrocarvone hydrochloride by sorption, which, however, was not found possible. Sorptogram: 1.4780_{US} 1.4762_1 1.4760_2 1.4762_3 1.4727_4 1.4613_5. The carefully distilled hydrochloride was converted to carone and distilled to give a main fraction: $1.4760_{US.\ 1-3}$ 1.4759_{4-5} 1.4750_6 1.4608_7. This fraction was treated with a saturated solution of $KMnO_4$ and $NaHCO_3$ while cooling to give the sorptogram 1.4760_{US} 1.4762_1 1.4760_2 1.4758_{3-7}. After a large sorption, a middle fraction of refractive index 1.4758 was obtained which gave the micro sorptogram 1.4751_1 1.4752_2 1.4754_3 1.4756_4 1.4758_{5-6} 1.4624_7. However, repeated distillation of the permanganate sample gave a product $1.4758_{US.\ 1-5}$ 1.4759_{6-7} which was used for the study of mixtures.

Carone regenerated from the semicarbazone was distilled to give a sorptogram of the main fraction 1.4738_{US} 1.4711_1 1.4725_2 1.4738_3 1.4741_4 1.4747_5 1.4751_6 1.4722_7, which demonstrates that decomposition had taken place. Repeated micro sorption, where the higher fractions were combined to give 40 μl, gave a new sorptogram, though of insufficient purity, 1.4753_1 1.4758_2 1.4762_3 1.4763_4 1.4762_5 1.4732_6 1.4540_7.

Mixtures of the ketones

The mixtures were prepared with the aid of small capillary pipettes, usually in about 50 to 70 μl quantities, which was sufficient to fill the micro pipette with 40 μl

without having trouble with air bubbles in the pipette. The accuracy of the composition of the mixture was of the order of 5%. This was acceptable since the sorptograms of the mixtures are given to demonstrate the separation caused by the gel in the range of the compositions indicated. The samples of limonene containing small parts of carvone were prepared by weighing in small vessels.

Carvone–carone

12 % carvone
1.4778_1 1.4782_2 1.4787_3 1.4788_4 1.4791_5 1.4793_6 1.4758_7

50 % carvone
1.4860_1 1.4861_{2-5} 1.4862_6 1.4864_7

88 % carvone
1.4942_{1-3} 1.4941_{4-5} 1.4939_{6-7}

Carvone–carvenone

47 % carvone
1.4888_1 1.4887_2 1.4886_3 1.4880_4 1.4878_5 1.4874_6 1.4806_7

Carvone–carvotanacetone

25 % carvone
1.4817_1 1.4821_2 1.4827_3 1.4830_4 1.4832_5 1.4838_6 1.4840_7

47 % carvone
1.4854_1 1.4860_2 1.4865_3 1.4870_4 1.4873_5 1.4878_7 1.4882_7

70 % carvone
1.4901_1 1.4904_2 1.4908_3 1.4912_{4-5} 1.4913_6 1.4915_7

Carvone–cis-*dihydrocarvone*

11 % carvone
1.4704_1 1.4710_2 1.4717_3 1.4727_4 1.4733_5 1.4739_6 1.4750_7

50 % carvone
1.4788_1 1.4803_2 1.4818_3 1.4828_4 1.4838_5 1.4844_6 1.4853_7

88 % carvone
1.4920_1 1.4928_2 1.4932_3 1.4937_4 1.4939_5 1.4942_6 1.4948_7

Carvone–trans-*dihydrocarvone*

12 % carvone
1.4688_1 1.4691_2 1.4699_3 1.4708_4 1.4720_5 1.4738_6 1.4769_7

48 % carvone
1.4760_1 1.4782_2 1.4802_3 1.4818_4 1.4830_5 1.4848_6 1.4858_7

88 % carvone
1.4911_1 1.4921_2 1.4928_3 1.4932_4 1.4938_5 1.4941_6 1.4947_7

Carvone–eucarvone

27 % carvone
1.5058_1 1.5052_2 1.5048_3 1.5039_4 1.5033_5 1.5028_6 1.4972_7

50 % carvone
1.5031_1 1.5023_2 1.5021_3 1.5017_4 1.5011_5 1.5007_6 1.4886_7

76 % carvone
1.5010_1 1.5001_2 1.4998_3 1.4994_4 1.4991_5 1.4988_6 1.4986_7

Carvone–menthone

51 % carvone
1.4578_1 1.4641_2 1.4701_3 1.4742_4 1.4780_5 1.4807_6 1.4804_7

Carvone–limonene

1 % carvone
1.4703_{1-7}

2 % carvone
1.4703_{1-6} 1.4708_7

12 % carvone
1.4703_{1-5} 1.4712_6 1.4858_7

50 % carvone
1.4703_{1-3} 1.4807_4 1.4907_5 1.4935_6 1.4948_7

88 % carvone
1.4834_1 1.4921_2 1.4939_3 1.4950_4 1.4954_5 1.4961_6 1.4967_7

Carone–carvotanacetone

42 % carone
1.4776_1 1.4773_{2-3} 1.4772_{4-6} 1.4722_7

Carone–trans-*dihydrocarvone*

12 % carone
1.4682_1 1.4686_2 1.4688_3 1.4690_4 1.4692_5 1.4698_6 1.4658_7

50 % carone
1.4710_1 1.4716_2 1.4718_3 1.4720_4 1.4722_5 1.4723_6 1.4708_7

88 % carone
1.4748_{1-3} 1.4749_{4-6} 1.4632_7

Carvenone–carvotanacetone

53 % carvenone
1.4789_1 1.4792_2 1.4793_3 1.4797_4 1.4748_5 1.4799_6 1.4720_7

*Carvotanacetone–*cis*-dihydrocarvone*

49 % carvotanacetone
1.4736_1 1.4739_2 1.4740_3 1.4741_4 1.4742_5 1.4741_6

*Carvotanacetone–*trans*-dihydrocarvone*

49 % carvotanacetone
1.4727_1 1.4730_2 1.4732_3 1.4734_4 1.4739_5 1.4740_6 1.4639_7

Carvotanacetone–eucarvone

53 % carvotanacetone
1.4941_1 1.4923_2 1.4917_3 1.4909_4 1.4899_5 1.4892_6 1.4732_7

Carvotanacetone-menthone

51 % carvotanacetone
1.4567_1 1.4598_2 1.4622_3 1.4638_4 1.4658_5 1.4669_6 1.4558_7

cis-*Dihydrocarvone–*trans*-dihydrocarvone*

10 % cis-dihydrocarvone
1.4681_{1-2} 1.4683_{3-4} 1.4684_5 1.4685_6 1.4687_7

50 % cis-dihydrocarvone
1.4681_1 1.4688_2 1.4690_3 1.4691_{4-5} 1.4692_6 1.4693_7

trans-*Dihydrocarvone–eucarvone*

54 % dihydrocarvone
1.4882_1 1.4873_2 1.4870_3 1.4863_4 1.4858_5 1.4851_6 1.4841_7

trans-*Dihydrocarvone–menthone*

47 % dihydrocarvone
1.4542_1 1.4559_2 1.4573_3 1.4589_4 1.4601_5 1.4615_6 1.4578_7

Eucarvone–menthone

43 % eucarvone
1.4629_1 1.4670_2 1.4701_3 1.4731_4 1.4758_5 1.4782_6 1.4783_7

Isomerizing effect of the gel

Small samples, 80 μl, of ketones were mixed with ~ 5 mg of activated gel in small tubes which were sealed. The ampoules were heated at 100°C for 18 hours and the ketones were then analysed by sorption to give the following sorptograms:

Carvone: $1.4969_{US,\ 1-4}$ 1.4970_5 1.4972_6 1.4960_7.
Eucarvone: 1.5064_{US} 1.5072_{1-6} 1.5069_7.
Carone: 1.4740_{US} 1.4763_1 1.4762_{2-4} 1.4750_5 1.4637_6.

Effect of storing

The sorptograms given below demonstrate the effect of storing some index-homogeneous samples in airtight, sealed ampoules at room temperature. The ketones were sealed immediately after their preparation in August 1956 and not opened until they were analysed again in January 1957.

Carvenone: 1.4809_{US} 1.4803_1 1.4809_{2-3} 1.4811_4 1.4812_{5-6} 1.4718_7.
Carvotanacetone: 1.4789_{US} 1.4786_1 1.4788_2 1.4789_{3-5} 1.4778_6 1.4629_7.
cis-Dihydrocarvone: 1.4682_1 1.4691_{2-3} 1.4692_4 1.4693_5 1.4699_6 1.4670_7.

Autoxidation of (+)*-carvone*

1 g pure carvone was stored in a flask which was connected to a rubber balloon filled with oxygen at atmospheric pressure. The oxidation was performed at room temperature and in diffuse daylight. Small samples were taken out at the intervals given and analysed by sorption.

6 days: 1.4968_{US} 1.4961_1 1.4964_2 1.4969_{3-7}.
13 days: 1.4969_{US} 1.4978_1 1.4970_{2-5} 1.4971_6 1.4972_7.
22 days: 1.4970_{US} 1.4982_1 1.4973_2 1.4972_{3-5} 1.4978_6 1.4979_7.
27 days: 1.4972_{US} 1.4990_1 1.4982_2 1.4972_3 1.4976_4 1.4978_{5-6} 1.4958_7.
37 days: 1.4980_{US} 1.4993_1 1.4990_2 1.4988_3 1.4978_4 1.4979_{5-6} 1.4610_7.

Effect of height of analytical column

In all work done by the present authors, we have used the size of the analytical column given originally by Blohm (2). The few experiments given below show that there is no further separation of carvone–carone mixtures with increased column length (50 cm) but that there is a difference between 12.5, 25 and 50 cm columns for analytical purposes for e.g. carvone–menthone mixtures.

Carvone–carone, 50%; $1.4869_{US.\ 1-6}$ 1.4867_7 1.4860_8.

59% carvone in menthone:

12.5 cm column: 1.4659_1 1.4699_2 1.4727_3 1.4752_4 1.4781_5 1.4798_6 1.4687_7.
25 cm column: 1.4608_1 1.4671_2 1.4730_3 1.4768_4 1.4801_5 1.4823_6 1.4688_7.
50 cm column: 1.4559_1 1.4640_2 1.4692_3 1.4758_4 1.4802_5 1.4839_6 1.4859_7.

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Enthalpy titrations involving citrate and tartrate chelates

By Joseph Jordan[1] and M. P. Ben-Yair[2]

With 5 figures in the text

The designation "Enthalpy titrations" has recently been proposed for methods based on the plot of temperature change versus mole ratio of reactants, in a titration carried out under judiciously controlled adiabatic conditions [1, 2]. Terms such as "thermometric titrations" [3] and "thermal titrations" [4] have at times been used in the literature for investigations of this type, a comprehensive review of which is contained in an article by Linde, Rogers and Hume [3]. The principal distinctive characteristic of enthalpy titrations is that they depend on the heat of the reaction *in toto* in accordance with the equation

$$\Delta H = \Delta F + T\Delta S. \qquad (1)$$

Because of the entropy term in equation (1), it was anticipated that enthalpy titrations may be applicable to situations where methods based solely on free energy (e.g. potentiometry) are liable to fail [1, 2].

Specifically, the purpose of the investigation reported in this paper was to explore the applicability of enthalpy titrations to analytical and structural studies of citrate and tartrate complexes. The interaction of citrate and tartrate with several divalent cations was selected as the subject matter of this study, because it warrants considerable fundamental interest. Citrate and tartrate chelates have been used extensively in analytical chemistry, although many of the relevant structures are still controversial.

Experimental

Materials: C. P. chemicals were used throughout.

Procedure: The enthalpy titrations were carried out with the aid of an exploratory non-automatic discontinuous "point by point" thermometric technique, which was adapted from methods described by various authors [5, 6, 7, 8]. Two solutions (e.g. trisodium citrate or disodium tartrate on the one hand, and the cation on the other) were allowed to react in a thermally insulated vessel and the concomitant change in temperature was recorded. One of the solutions was titrated into the other in a Dewar flask and the temperature in the latter was read with the aid

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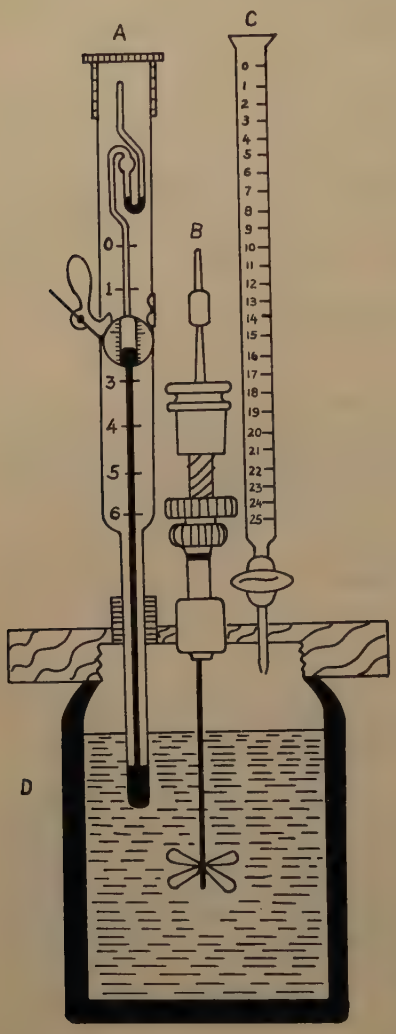


Fig. 1. Cross section of titration apparatus. *A*, Beckmann thermometer; *B*, stirrer; *C*, burette; *D*, titration cell (Dewar flask).

of a Beckmann thermometer. The corresponding experimental set-up is shown in Fig. 1.

In each titration 200 ml of solution, in a range of concentrations between 0.01 and 0.05 *M*, were titrated from a 25 ml buret with a 1.00 *M* aqueous reagent solution. Thus the milliliters added at any point of the titration represented millimoles of titrant. In the enthalpy titration curves, these were plotted on the abscissa versus Beckmann readings on the ordinate. Pure aqueous solutions were titrated, as well as some solutions in ethanol–water mixtures. The titrants were always added at a constant rate of 1 ml/min and kept at room temperature (25° ± 3°C). The initial temperature of the solutions in the Dewar was adjusted to about 1°C below that of the corresponding titrant.

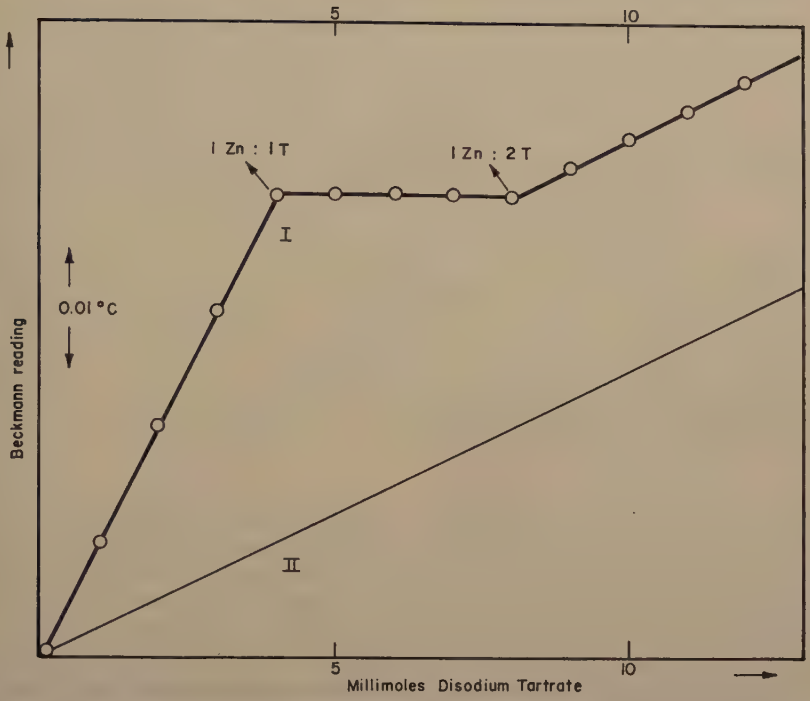


Fig. 2. Enthalpy titration of zinc with tartrate. I, Titration curve of 200 ml 0.02 *M* aqueous Zn^{++} solution with 1 *M* disodium tartrate. II, Blank: titration of 200 ml water with 1 *M* disodium tartrate.

Results

A typical enthalpy titration curve is plotted in Fig. 2, Curve I, involving chelation of zinc and tartrate ions. An aqueous solution of zinc nitrate was titrated with disodium tartrate. Precipitation did not occur under the experimental conditions. As can be seen in the figure, the titration curve shows three discrete rectilinear portions, the last of which is parallel to a straight line obtained in a "blank titration" of distilled water with disodium tartrate (Fig. 2, Curve II). The two breaks in Curve I, *A* and *B*, are interpreted as corresponding to the formation of the complexes [ZnT] and $[ZnT_2]^{--}$, respectively, the symbol T being used to denote the anion $\bar{O}OC–CHOH–CHOH–C\bar{O}\bar{O}$. In contradistinction to zinc, one significant break only was obtained in enthalpy titrations of cupric ion with disodium tartrate. This was taken to indicate that in the corresponding cupric tartrate complex the stoichiometric ratio was 1:1 between cupric ion and the chelating agent. Identical conclusions have been derived from independent electrometric and photometric studies [9]. Consequently it appears warranted to infer that the breaks in the enthalpy titration curve are analytically and stoichiometrically significant. Experimental work involving citrate on the one hand, and several divalent cations on the other [cf. 9], indicated a similar significant agreement between enthalpy titrations and conventional methods.

Representative comparable results are summarized in Table 1.

Table 1. Results of enthalpy titrations with disodium tartrate and trisodium citrate.[a]

Cation[b] titrated	Behavior versus tartrate[c]		Behavior versus citrate[d]	
	No of significant breaks on curve	Stoichiometric composition of corresponding chelates	No. of significant breaks on curve	Stoichiometric composition of corresponding chelates
Cu(II)	1	$[CuT]^0$	1	$[CuCit]^-$
Zn	2	$[ZnT]^0$; $[ZnT_2]^{--}$	1	$[ZnCit]^-$
Cd	1	$[CdT]^0$	1	$[CdCit[^-$

[a] The ligands were added as titrants in 1 *M* solutions.
[b] 200 ml of 0.02 *M* solution were titrated in each experiment.
[c] "T" denotes the divalent tartrate anion.
[d] "Cit" denotes the trivalent citrate anion.

Previous physico-chemical studies have indicated that citrate and tartrate chelates may interact with alkalis as though they were monoprotic and diprotic acids [9]. It was anticipated that enthalpy titrations may be ideally suited for studying the stoichiometry of the relevant neutralization phenomena, because of the relatively large enthalpies involved [3]. In the corresponding experiments, dilute solutions (0.02 *M*) of citrate and tartrate chelates were titrated with sodium hydroxide or ammonia (ammonia was used in instances when sodium hydroxide yielded precipita-

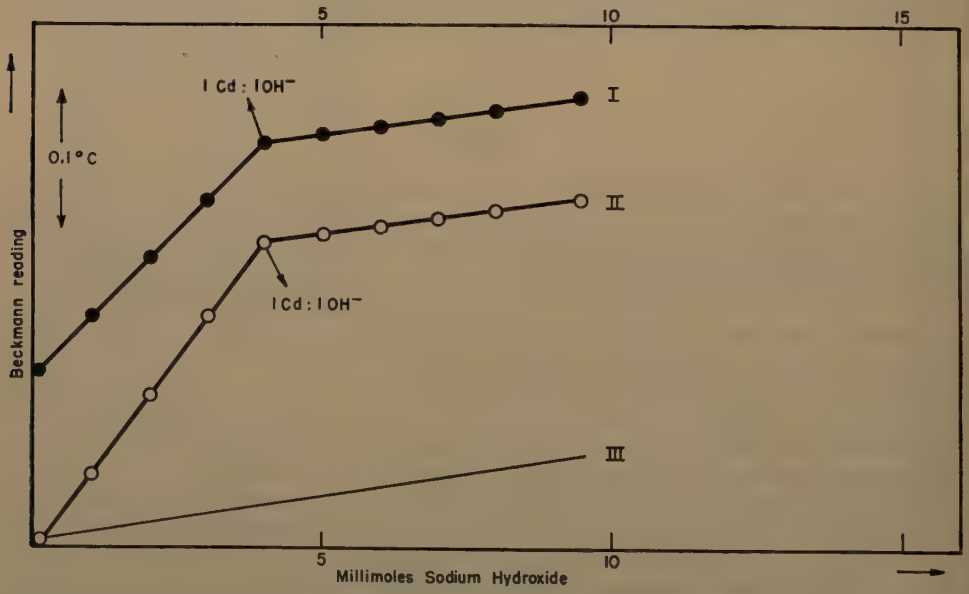


Fig. 3. Enthalpy titration curves with alkali of cadmium chelates. I, 200 ml of 0.02 *M* aqueous cadmium tartrate plus 0.02 *M* excess disodium tartrate titrated with 1 *M* NaOH. II, 200 ml of 0.02 *M* aqueous cadmium citrate plus 0.02 *M* excess trisodium citrate titrated with 1 *M* NaOH. III, Blank: 200 ml water titrated with 1 *M* NaOH.

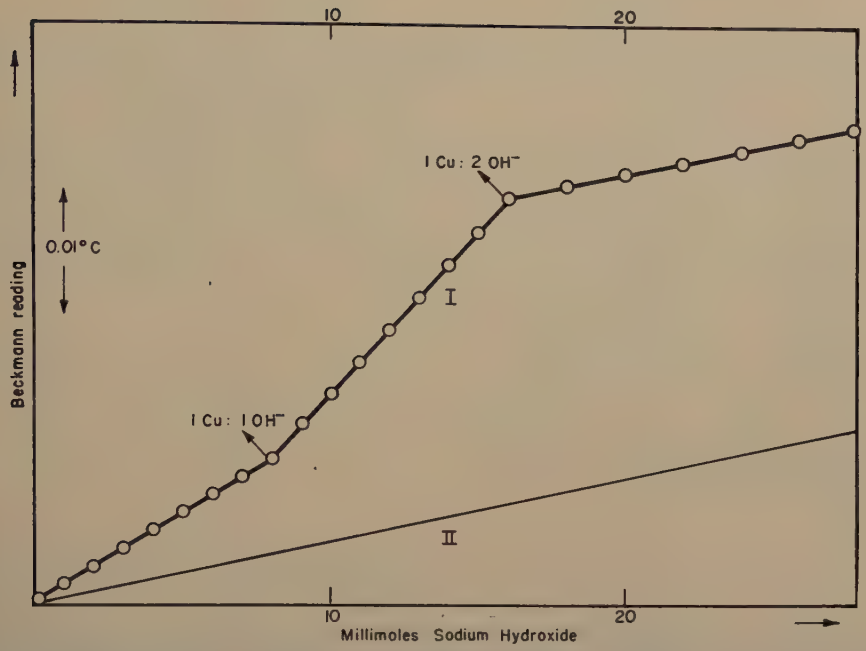


Fig. 4. Enthalpy titration of cupric tartrate chelate. Solvent: 50 % EtOH–50 % water mixture (v/v). I, 200 ml 0.04 *M* cupric tartrate titrated with 1 *M* NaOH. II, Blank: 200 ml solvent titrated with 1 *M* NaOH.

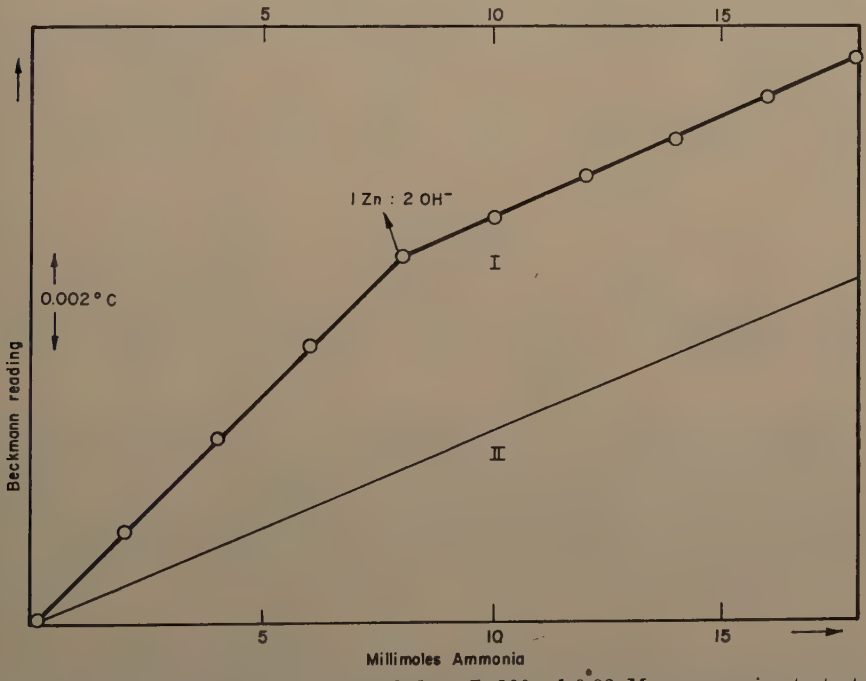


Fig. 5. Enthalpy titration of zinc tartrate chelate. I, 200 ml 0.02 *M* aqueous zinc tartrate plus 0.02 *M* excess disodium tartrate titrated with 1 *M* NH_4OH. II, Blank: 200 ml water titrated with 1 *M* NH_4OH.

Table 2. Characteristics of alkalimetric enthalpy titration curves.[a]

Chelate		Breaks in enthalpy titration curves, corresponding to	
Stoichiometric composition	Acid-base characterization	First ionizable proton	Second ionizable proton
$[CuT]^{o}$ [b,c]	Diprotic acid	+	+
$[CuCit]^{-}$ [d]	Monoprotic acid	+	
$[ZnT_2]^{--}$ [c]	Diprotic acid	−	+
$[ZnCit]^{-}$ [d]	Monoprotic acid	+	
$[CdT]^{o}$ [c]	Monoprotic acid	+	
$[CdCit]^{-}$ [d]	Monoprotic acid	+	

[a] Obtained in 0.02 *M* solutions of various chelates.
[b] Titrated in 50 % EtOH.
[c] Same footnote as in Table 1.
[d] Same footnote as in Table 1.

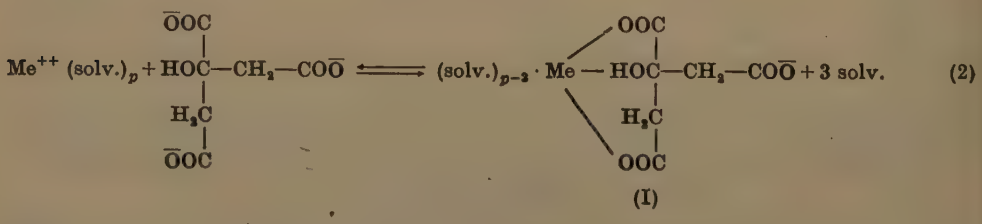
tion). The titrations with alkali were carried out either in aqueous solutions, in which case an appreciable excess of ligand was added in order to stabilize the various complexes; or a 50 % EtOH–50 % water mixture (v/v) was used as solvent, which has been found to satisfactorily repress dissociation of the chelates when the solution contained stoichiometric amounts of cations and ligands [cf. 9]. Typical alkalimetric enthalpy titration curves are shown in Figs. 3, 4, and 5.

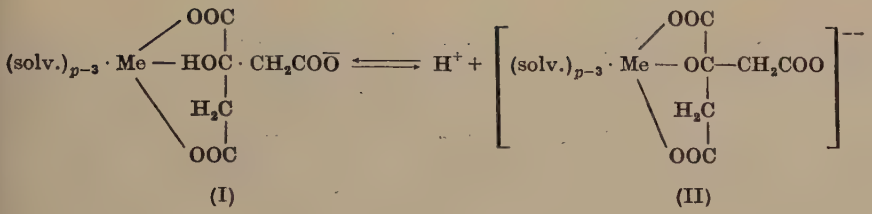
Fig. 3 illustrates the behavior of a chelate (cadmium citrate) which reacted with alkali as a monoprotic acid. Fig. 4 indicates the behavior of a cupric tartrate chelate which yielded two discrete breaks corresponding to the successive neutralization of two acid hydrogens. Fig. 5 (zinc tartrate) represents an example of a neutralization curve exhibiting one break only, corresponding stoichiometrically to the neutralization of a diprotic acid. A complete summary of the relevant stochastic data is given in Table 2.

Discussion

Results reported in this paper can satisfactorily be accounted for on the basis of the following equilibria, involving a divalent cation (denoted by Me^{++}) and citrate or tartrate ligands.

For citrate:

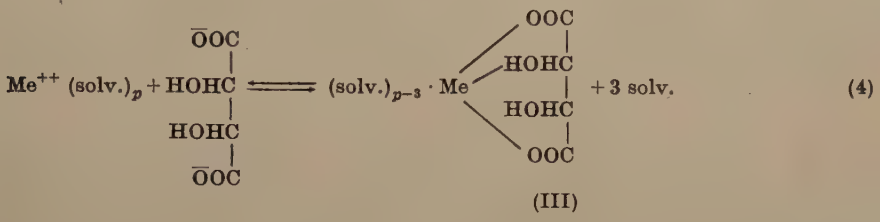




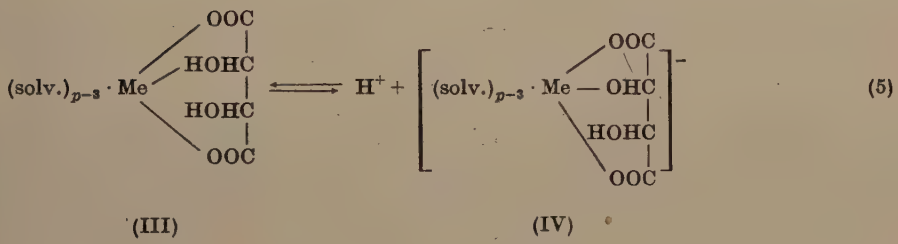
(I) (II) (3)

where "solv." denotes solvated molecules and p is the effective coordination number of the relevant cation.

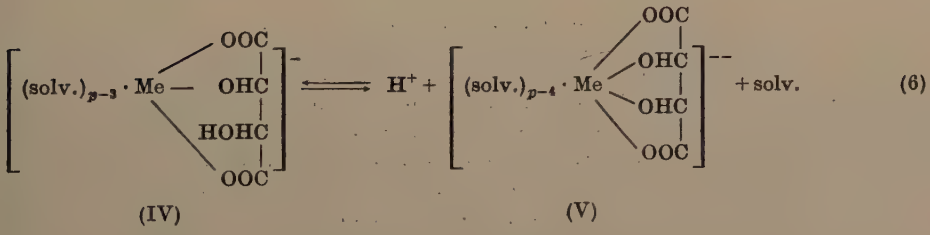
For tartrate:



(III) (4)



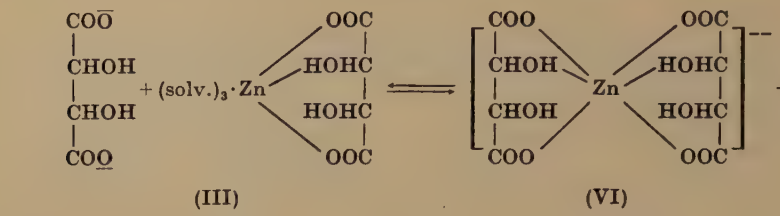
(III) (IV) (5)



(IV) (V) (6)

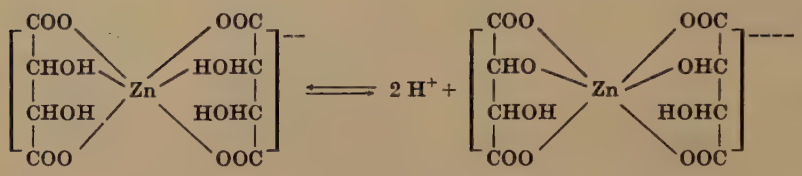
Prevalence of equilibria of the type indicated by equations (2) and (4) has been amply confirmed by electrochemical and optical studies [9]. The unsymmetrical structure (III) has recently been established unequivocally for cupric tartrate [10] by infrared spectrum investigations. Equation (3) accounts for the behavior of the citrate chelates as monoprotic acids. Equations (5) and (6) indicate that the tartrate chelates (III) may react as monoprotic or as diprotic acids, as was found experimentally.

To account for the reactions involving zinc and tartrate, the following sequence of additional equilibria is postulated.



(7)

(III) (VI)



(8)

(VI) (VII)

Naturally, a detailed consideration of Equilibrium 8 involves two successive ionization steps. However, the experimental results indicate that the relevant heats of reaction are not sufficiently dissimilar to yield in the enthalpy titration curve (Fig. 5) a discrete break corresponding to the first neutralization step. This is quite plausible in view of the symmetrical structure of species VI and VII and the analogous situation of the two protogenic hydroxyl groups in VI.

The structures postulated for the various citrate and tartrate chelates are considered reasonable and in accordance with currently accepted views of complex chemistry [11]. In each structure, the cation is part of a multiple ring system consisting of six-membered and/or five-membered rings. Fischer–Hirschfelder models of the various chelates appear to be well in line with modern concepts of bond lengths and configuration.

Analytical applications of enthalpy titrations

The well defined stoichiometric end-points in enthalpy titrations invite analytical applications. However, the discontinuous manual technique described in this paper is cumbersome and outdated. In recent years, automatic procedures have been developed for thermochemical titrations, based on motor-driven burettes synchronously coupled with a thermistor bridge circuit and a recording potentiometer. Under rigorously controlled experimental conditions, a method has been developed which is believed to be the first successful titrimetric determination of heats of reaction in dilute aqueous solutions [1]. An active research program is now being pursued at The Pennsylvania State University by one of the authors and by Mr. W. H. Dumbaugh [12], involving the determination by enthalpy titrations of weak acids and bases and the study of the relevant entropies of ionization.

A few selected examples of applications of manual enthalpy titrations to the determination of citrates and tartrates are reported below, in order to illustrate their potentialities and limitations. *Mutatis mutandis*, it is anticipated that they can be adapted to automatic instrumentation and acquire analytical significance, because of their rapidity, sensitivity and convenience.

Direct determination of citrate and tartrate was carried out by enthalpy titration, using 0.1 *M* cupric, cadmium and zinc nitrates as titrating reagents. The amount of citrate or tartrate was computed from the breaks on the titration curves and the stoichiometric composition of the complexes. Samples of approximately 200 ml disodium tartrate and trisodium citrate, about 0.02 *M*, were titrated. The relevant results are summed up in Table 3. The precision was computed as the standard deviation from the average of 10 titrations; the accuracy was estimated by comparison with a pentabromoacetone procedure [8] for citrate and by Ce(IV)-oxidimetry for tartrate [9].

Table 3. Determination of citrate and tartrate by enthalpy titration.

Reagent	Citrate determination		Tartrate determination	
	Precision %	Accuracy %	Precision %	Accuracy %
Cupric nitrate	2	2	0.5	0.7
Zinc nitrate	1	2	0.5	1 [a]
			1	2 [b]
Cadmium nitrate	0.5	3	2	2

[a] first break.
[b] second break.

Cupric ion appears to be altogether the most satisfactory analytical reagent among the three cations investigated. The tartrate determination is of better inherent precision and accuracy than the titration of citrate. The method is restricted to samples of pure tartrate and citrate and *cannot* be used for mixtures.

To analyze mixtures of tartrates and citrates the following indirect enthalpy titration method was used. First, the mixed sample was titrated with cupric nitrate. A break was obtained at a point corresponding to the sum of tartrate and citrate. Subsequently, another sample was treated with an excess of cupric nitrate and titrated with sodium hydroxide. A break was obtained at the point where cupric hydroxide starts separating. The equivalents of sodium hydroxide at this point correspond to the moles of citrate plus *twice* the number of moles of tartrate present. From the combined results of the two titrations the amounts of citrate and of tartrate were computed. Typical results are shown in Table 4.

Table 4. Determination of citrate and tartrate in mixtures.

Ratio citrate/tartrate	Accuracy of citrate determination %	Accuracy of tartrate determination %
50 : 1	2	6
10 : 1	2	4
1 : 1	2	1
1 : 10	3	1
1 : 50	10	1

Accuracies were estimated, in this case, by determining citrate and tartrate separately, prior to mixing. As can be seen in the table, the indirect (double titration) method is of poor accuracy. It might still prove useful, however, because other commonly used methods are equally unsatisfactory for the determination of citrate and tartrate in mixtures. *Ceteris paribus*, enthalpy titrations appear to have the advantage of being appreciably more rapid and convenient than the conventional methods.

Summary

Structural and anlytical studies of citrate and tartrate chelates are described, by a method called "Enthalpy titration". The method is based on a plot during a titration of temperature change in an adiabatic system versus mole ratio of reactants. The interaction of citrate and tartrate ligands has been investigated with zinc, cadmium and cupric ions. Enthalpy titrations indicated the formation in dilute solutions of various citrate and tartrate chelates, in excellent agreement with stoichiometric relationships inferred on the basis of electrochemical and optical investigations. The chelates reacted with alkali as monoprotic or diprotic acids. Reasonable structures are postulated accordingly and the relevant equilibria are presented and discussed. Exploratory application is described of enthalpy titrations for the quantitative determination of citrate and tartrate.

The significance of the enthalpy titration method is discussed in the light of its fundamental interest, inherent in its direct dependence on an entropy term.

A partial resumé of the contents of this paper was presented at the XIIth International Congress of Pure and Applied Chemistry, New York, 1951, [14].

Some of the experimental material is based on a thesis by M. P. Ben-Yar (Seinfeld), submitted in 1947 to the Hebrew University, Jerusalem, Israel, in partial fulfillment of the requirements for the degree of M.Sc. [13].

ACKNOWLEDGEMENT

The authors want to express their gratitude to the President of the Weizmann-Gothenburg Committee, Professor J. Arvid Hedvall, for his great help and interest in our work, part of which was carried out by Dr. Ben-Yair in the Institute for Silicate Research, Gothenburg.

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Tryckt den 15 april 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 11 nr 26

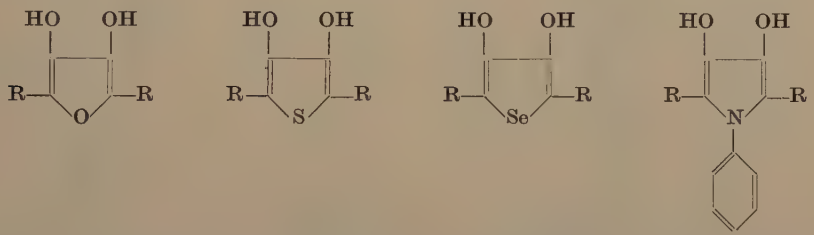
Mitgeteilt am 14. November 1956 durch H. von Euler

3,4-Dihydroxy-pyrrol-dicarbonsäure-(2,5)-dimethylester Darstellung und Eigenschaften

Von Hans Wåhlstam

Mit 1 Figur im Text

Bisreduktone (1), die durch ihre zwei Endiolgruppen zwei Mole Tillmans-Reagens (TR) zu reduzieren vermögen, sind gewonnen worden durch hydrolytische Aufspaltung von Hetero-Ringsystemen des Furans, Thiophens, Selenophens und N-Phenylpyrrols, und zwar der 3,4-Dihydroxy-derivate der 2,5-Dicarbonsäureester ($R = CO_2 . CH_3$).



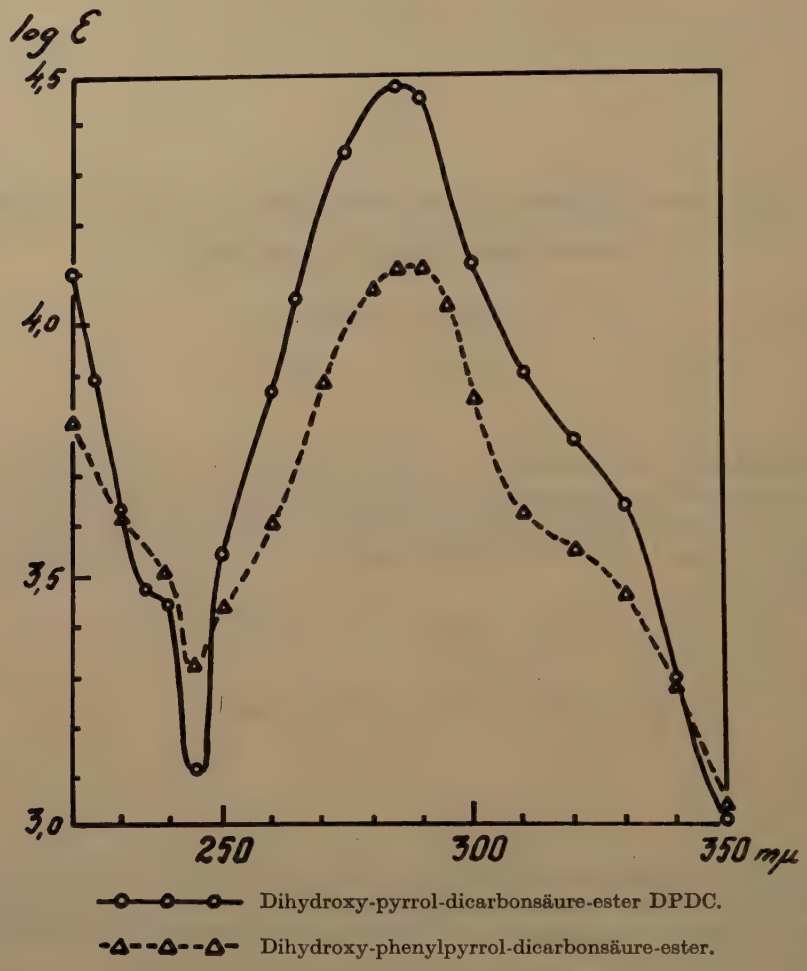
Die dem 3,4-Dihydroxy-N-phenylpyrrol-dicarbonsäure-(2,5)-dimethylester entsprechende phenylfreie Verbindung DPDC war noch nicht bekannt. Dieselbe kennen zu lernen erschien wünschenswert in Hinsicht auf die interessante, bifunktionelle Rolle der Bisreduktone.

Zur Darstellung von DPDC habe ich im wesentlichen den gleichen Weg eingeschlagen, auf dem die entsprechende Phenyl-pyrrol-Verbindung gewonnen worden war. Als Ausgangsmaterial stellte man Iminodiessigsäure-dimethylester dar, der mit Äthyloxalat in methylalkoholischem Natrium kondensiert wurde. Das so erhaltene, aus Methanol umkrystallisierte Produkt DPDC schmilzt bei 207°.

Die Analyse ergab die erwartete Zusammensetzung $C_8H_9O_6N$ (Mol.Gew. 215). Der Titration mit Tillmans-Reagens zufolge enthält DPDC per Mol *eine* Endiol-Gruppe.

Wie frühere Versuche (1) ergeben hatten, wird das N-Phenyl-pyrrol-derivat durch Alkali unter Bildung des Bis-endiols leicht aufgespalten, und es war zu erwarten, dass sich DPDC ebenso verhält. Dies ist *nicht der Fall*. Nach ½stündiger Einwirkung von 2-norm. Alkali tritt keine mit einer Ringöffnung verbundene zweite Endiolbildung ein, während in saurer Lösung (pH 4) sofort 1 Mol TR unter Bildung einer Endiol-Gruppe reduziert wird (vgl. S. 253).

Bei einem papierchromatographischen Versuch mit DPDC in n-Butanol gesättigtem Wasser und mit TR (Dichlorphenol-indo-phenol) in alkoholischer Lösung als



Entwickler trat nur *ein* Flecken, $R_f = 0{,}67$, auf, somit kein Anzeichen für die Bildung eines Bis-Reduktons oder eines anderen Produktes.

Das UV-Spektrum der Sbst. DPDC ist in der Fig. mit demjenigen des entsprechenden N-pehnyl-pyrrol-dicarbonsäure-esters zusammengestellt. Man erkennt die übereinstimmende Lage der den Pyrrolring kennzeichnenden Maxima, 286 mμ und Minima, 245 mμ.

Der Zusammensetzung und den übrigen Versuchsergebnissen zufolge kommt für DPDC das folgende Strucktur-Gleichgewicht in Betracht:

a *b*

Die Struktur *a* bringt zwar den Endiol- bzw. Redukton-Charakter der Sbst. DPDC zum Ausdruck. Was die Struktur *b* betrifft, so liegt meines Wissens bis jetzt noch keine genügende Analogie dafür vor, dass ein solcher Stoff TR so schnell reduziert wie DPDC. Der Befund, dass DPDC im Gegensatz zu dem enstprechenden Phenyl-pyrrol-dicarbonsäure-ester durch Alkali nicht unter Ringöffnung ein Bis-Redukton liefert, ist eine noch unerklärte, bemerkenswerte Tatsache.

Beschreibung der Versuche

Darstellung des 3,4-Dihydroxy-pyrrol-dicarbonsäure-(2,5)-dimethylesters

a) Semicarbazino-diessigsäure (2). Zu der heissen Lösung von 130 g Hydrazinsulfat in 500 ml Wasser wurden 69 g (0,5 Mol) Kaliumcarbonat in kleinen Portionen zugesetzt. Zu der abgekühlten Lösung wurden 81 g KCNO (1 Mol) so vorsichtig zugegeben, dass eine Temperatursteigerung vermieden wurde. Nach 10–12 Stunden fiel der grösste Teil des entstandenen Kaliumsulfates aus und wurde abfiltriert. Nach Berechnung enthielt die Lösung dann 80 % der theoretischen Ausbeute an Semicarbazid. Eine wässrige Lösung von 227 g (3 Mol) Monochloressigsäure wurde mit 165, 5 g (1,5 Mol) Kaliumcarbonat neutralisiert und mit der Semicarbazidlösung gemischt. Nach 12-stündigem Erhitzen auf dem Wasserbad wurde die Lösung im Vakuum zur Trockne eingedunstet.

b) Die so entstandene Mischung von Semicarbazino-diessigsäure-dimethylester und Kaliumsulfat wurde durch Erwärmen mit 300 ccm methylalkoholischer Salzsäure in Lösung gebracht und stand 1 Woche bei Zimmertemperatur. Dann wurden die anorganischen Salze abfiltriert und die alkoholische Lösung wurde mit einem Überschuss vom Ammoniak vesetzt. Der Alkohol wurde hierauf im Vakuum vertrieben, der Rückstand in wenig Wasser gelöst und mit Chloroform extrahiert. Aus diesem Chloroformextrakt erhielt man nach Entfernung des Chloroforms den Ester. Ausbeute 35,2 g Sbst. vom Schmpt 143°. Man liess nun 5,0 g dieses Esters + 6,3 g Natriumnitrit + eine äquivalente Menge konc. Salzsäure in wässriger Lösung reagieren, wobei sich die Lösung sofort stark gelb färbte und eine langsame Entwicklung von CO_2 und Stickoxyden einsetzte. Nach Beendigung dieser Gasentwicklung war die Lösung fast farblos. Der gebildete Iminodiessigsäure-dimethylester wurde mit Äther extrahiert, der Ester bei 123,5°, 16 mm Hg destilliert (3). Ausbeute 2,2 g.

c) Schliesslich wurde 1 g Imino-diessigsäure-dimethylester mit 1,0 g Äthyloxalat vermischt und mit einer Lösung von 0,5 g Natrium in 10 ml Methylalkohol versetzt, wobei sich fast unmittelbar ein grünschwarzes Gel bildete. Nach Stehen über Nacht wurde die Lösung des Gels in 25 ml Wasser angesäuert und wiederholt mit Äthylacetat augeschüttelt. Die mit Natriumsulfat getrocknete Lösung wurde im Vakuum zur Trockne eingedunstet. Der Rückstand wurde abfiltriert und gewaschen. Ausbeute 450 mg. Die erhaltene Substanz, DPDC, wurde aus Methanol umkrystallisiert. Schmpt 207°.

Analyse. Für $C_8H_9O_6N$	Ber. C 44,75 %	H 4,18 %	N 6,52 %
	Gef. 44,96 %	4,33 %	6,43 %

Endiol-Titration des Esters DPDC mit Tillmans-Reagens

4,7 mg DPDC, in 10 ml Wasser suspendiert, verbrauchten bei der Titration mit einer 0,0075-n. TR-Lösung 5,80 ml. Hieraus berechnet sich für die neutral DPDC-Lösung der Endiol-Gehalt 1,0.

Nach Zusatz von 5 ml 2-n. Natronlauge und ½stündigem Erwärmen auf dem Wasserbad unter Stickstoff verbrauchte die Lösung nur noch weitere 0,1 ml TR-Lösung. Durch die Erhitzung in alkalischer Lösung stieg also die Endiol-Menge nicht weiter.

Acetylierung des Pyrrol-dicarbonsäure-esters DPDV

50 mg des Esters wurden nach Zusatz von ½ ml Essigsäureanhydrid und einer Spur konz. Schwefelsäure unter Erwärmung auf dem Wasserbad in Lösung gebracht. Nach 10 Minuten wurde mit der doppelten Menge Wasser verdünnt. Das acetylierte Produkt wurde mit Äthylacetat extrahiert und krystallisierte aus der eingeengten Lösung in Nadeln aus. Die abgekühlten Krystalle wurden mit Äthylacetat gewaschen. Ausbeute an Rohprodukt 30 mg. Nach Umkrystallisieren aus Äthylacetat schmolz der acetylierte Pyrrol-dicarbonsäure-ester bei 135°. Die Zusammensetzung $C_{12}H_{13}O_8N$ zeigt, dass sich eine *Diacetyl*-Verbindung gebildet hat.

Analyse. Für $C_{12}H_{13}O_8N$ Ber. N 4,68 %
Gef. 4,85 %

Herrn Assistenten Ingenieur Hans Hasselquist danke ich bestens für seine wertvolle Hilfe bei der Ausarbeitung der vorliegenden Untersuchung.

Stockholm, Universität, Institut für organ.-chemische Forschung.

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ARKIV FÖR KEMI Band 11 nr 27

Communicated 10 April 1957 by Karl Myrbäck and Erik Jorpes

A method of assaying small amounts of S^{35} in tissues

By Claes-Henrik Dohlman

With 1 figure in the text

Most textbooks on tracer methodology (e.g. Hevesy 1948) recommend that S^{35} should preferably be determined as an "infinitely thick" layer of barium sulfate or benzidine sulfate. However, if the radioactivity in the sample is low, addition of enough unlabeled carrier sulfate to get an "infinitely thick" layer of barium sulfate will inconveniently decrease the count rate. Using thinner layers of barium sulfate involves considerable disadvantages. Thus, adding little or no carrier sulfate will jeopardize quantitative precipitation of S^{35}, especially from an acid solution. Another disadvantage is that weighing of small amounts of barium sulfate may involve a considerable error and make corrections for self-absorption inaccurate. It is also difficult to obtain an even distribution of a few milligrams of barium sulfate on a planchet; therefore self-absorption might vary significantly between samples of the same weight.

A simple method of determining total S^{35} in tissue specimens is presented here. The first step, wet combustion of the tissue, is similar to the combustion procedures used for various purposes by Pirie (1932), Seligman and Fine (1943), Boursnell, Francis and Wormall (1946), Cember, Watson and Grucci (1954) and Gedda (1956). The acid ash is then dissolved in water and transferred to lead planchets. The sulfate precipitates very evenly on the planchets and the radioactivity can be assayed with moderate error.

Method

The tissue specimens are transferred to 10 ml thick-walled test tubes with ground joints. 0.2 ml 2 per cent H_2SO_4 and 2 ml fuming HNO_3 are added. Reflux condensers are inserted into the glass joints of the test tubes and the tubes heated to 80°C in a temperature-regulated silicone oil bath. After half an hour the condensers are removed and the temperature raised to 110–120°C. When all fluid has evaporated, the test tubes are cooled and 2 ml of 30 per cent H_2O_2 added. The tubes with air condensers are again heated at 90°C during half an hour, and, without condensers, evaporated to dryness at 100–110°C.

The ash is dissolved in 2.0 ml distilled water, and 0.5 ml of the solution is pipetted to each of 3 lead planchets and evaporated at 40°C. If the radioactivity is low, the fluid volume might be less and only one planchet be used. A thin layer of silicone grease should have been smeared in advance on the outside edges of the

planchets in order to prevent "creeping" of the fluid during drying. A very thin, uniform layer of radioactive material is obtained. The count rate of the samples is then determined in a standard position under a thin-window G-M tube.

Results and discussion

The accuracy of the method was tested by combusting aliquots of an S^{35}-labeled sodium sulfate solution together with increasing amounts of freeze-dried bovine cornea. The result of such experiments is shown in the figure. The arithmetic mean of ten determinations without tissue was set at 100 per cent. One standard deviation of these data was only $\pm$ 2.1 per cent. When increasing amounts of tissue were added, the recovery of S^{35} was not significantly lowered until 90 mg

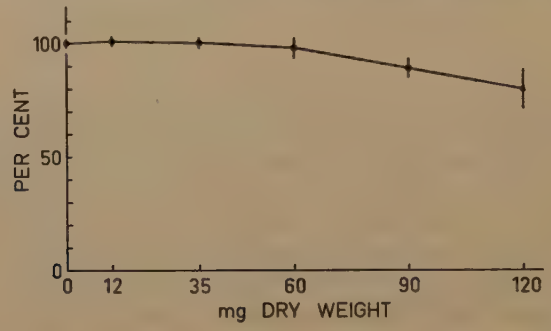

Fig. 1. Recovery of S^{35}-sulfate after combustion with increasing amounts of unlabeled dry cornea. The arithmetic mean $\pm$ one standard deviation $[s = \sqrt{\Sigma (X - m)^2 / (N - 1)}]$ is given for each series of six or ten determinations.

cornea was used. However, the error appeared to increase with the addition of tissue. For six or ten determinations with each of 12, 35, 60, 90 and 120 mg dry cornea, the value of one standard deviation was 2.6, 2.8, 4.4, 4.3 and 8.7 per cent, respectively (see figure). The standard error of the counting rate in these experiments was 1.1 per cent.

The recovery of S^{35} after the combustion procedures was compared with that after direct evaporation of the test solution on lead planchets. Combustion reduced the recovery to 74.4 per cent of the control value, most probably because of self-absorption on the planchets. The recovery could be increased to 82.0 per cent by not adding sulfuric acid prior to combustion, but then the errors increased considerably.

The sulfate of the ash was probably to a great extent precipitated as lead sulfate on the planchets. On titration the ash was found to contain enough acid to release more lead ions from the planchet surface than corresponded to sulfate and phosphate ions in 100 mg dry cornea. It is probable that the even precipitation of lead sulfate over the whole surface was the reason why the results differed only moderately. When planchets of aluminum, cupper, glass or steel were used, the data became widely scattered. Lead planchets have still another advantage in that they give high back-scatter and thereby increase the count rate.

This method seems convenient for determination of small amounts of total S^{35} (ester-sulfur as well as neutral sulfur). The weight of the tissue samples should preferably not exceed 60 mg dry weight. It is felt that this method is more simple and less time-consuming and still accurate enough compared with the determination of small amounts of S^{35} as barium or benzidine sulfate.

Summary

A method for the determination of S^{35} in tissues, convenient for serial analyses, is described. The tissue samples are combusted with nitric acid and hydrogen peroxide, the acid ash dissolved in water and directly transferred to lead planchets. The sulfate precipitates (probably as lead sulfate) as a thin and even layer, and the radioactivity can be measured with a fair degree of accuracy.

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Application of zone electrophoresis and cellulose ion exchange chromatography to the fractionation of posterior pituitary hormones

By Jerker Porath

With 6 figures in the text

In 1895 Oliver and Schäfer discovered that intravenous injection of a pituitary extract caused a *rise* in blood pressure in mammals (1). Eleven years later Dale discovered the uterine contracting principle (2). During the next few years the milk ejecting factor in mammals (Ott and Scott 1910 (3)) the *lowering* of blood pressure in birds (Paton and Watson 1912 (4)) and the antidiuretic effect (van den Velden 1913 (5)) became known. Thus at the beginning of this century the posterior lobe of the pituitary gland was known to be an important source of hormones. In spite of this fact about half a century passed before the isolation of well-defined chemical substances, having the properties mentioned, was achieved. In 1949 Livermore and du Vigneaud (6) isolated a polypeptide of bovine origin, oxytocin, which is responsible for all or at least the major part of the uterine contracting, milk ejecting and avian depressor activity of the posterior lobe. Two years later Turner, Pierce and du Vigneaud (7) isolated from the same source another related peptide, vasopressin, which possesses the greater part of the pressor and antidiuretic activity of the gland. In the short span of only four years the complete elucidation of the structures of bovine oxytocin (Tuppy (8), du Vigneaud *et al.* (9)) and bovine vasopressin (the school of du Vigneaud (10) and Acher and Chauvet (11)) was accomplished. Both hormones have now been synthesized (du Vigneaud (12, 13) and Boissonnas (14)).

The long lapse of time between the discovery of the endocrine functions and the isolation of the corresponding substances contrasted with the short time interval between isolation and syntheses clearly demonstrates the paramount importance of fractionation work in the chemistry of natural products.

The contributors in the field of isolation of oxytocin and vasopressin are numerous: Abel *et al.*, Kamm (18) the du Vigneaud school, van Dyke *et al.*, Potts and Gallagher, and quite recently Maier-Hüser, Fromageots and du Vigneaud's school. Excellent reviews have been published by Irving and du Vigneaud (15), Landgrebe, Ketterer and Waring (16) and du Vigneaud (17). A valuable review of early work and theories is given in the comprehensive paper of Kamm (18).

Since 1932 it has been known that the intermediate lobe of the pituitary also

contains a hormone. This was shown by Zondek and Krohn (19) in that an extract from the intermediate lobe darkens the skin of amphibia as a consequence of the dispersion of the pigment containing bodies of the skin. The hormone has been called intermedin, "B", melanophore expanding hormone, melanosome dispersing hormone and melanocyte stimulating hormone (MSH). The latter name will be used by the author. Reviews have been published by Waring *et al.* (20) and Landgrebe *et al.* (16).

Attempts to obtain potent preparations were made by Stehle in 1936 (21). It was not until fairly recently, however, that MSH received special attention. In connection with the extensive studies on ACTH it was noticed that the ascorbic acid depletion activity parallelled the melanocyte dispersion potency. Some investigators put forward the hypothesis that the two physiological activities could be attributed to a single substance only (Johnson and Högberg (22) and Sulman (23)). This could not be the case, however, as was demonstrated by Reinhardt *et al.* (24) and others. Landgrebe and Mitchell (25), using the oxycellulose procedure of Astwood *et al.* (26) on posterior lobe extract succeeded in obtaining very active concentrates of MSH.

In 1954 Lerner and Lee (27) claimed the isolation of a pure melanocyte stimulating peptide from swine. The present author and his collaborators (28) also announced the isolation of a homogeneous peptide causing dispersion of melanocytes. In spite of the fact that the peptides both had been isolated from swine they had very different properties. While the peptide of Lerner and Lee had an isoelectric point of pH 10.5–11, the isoelectric point of our peptide was found to be close to pH 5.0. A melanocyte stimulating peptide similar to or identical with ours was also isolated at about the same time by Benfey and Purvis (29). The basic melanocyte stimulating peptide has been called α-MSH and the other peptide β-MSH (30). β-MSH is composed of 18 amino acid residues as was shown by Roos (31). The amino acid sequence has been elucidated by Harris and Roos (32). It exhibits remarkable resemblance to that of corticotropin since these two peptides possess a sequence of 7 amino acid residues in common. Recently Geschwind *et al.* have isolated a peptide the composition of which was found to be identical with β-MSH (33). Two MSH present in pituitary glands of dogs have recently been demonstrated by Sulman and Aviatar (34).

Simultaneously with the isolation of β-MSH the electrophoretic methods, earlier discussed, were developed. In view of the increasing interest in posterior lobe hormones as a result of the discovery of their origin in the hypothalamus it appeared to the author that a broad investigation of the peptide content of the two organs might be of great value as a complement to physiological and anatomic investigations.

The author has made recent studies partly in collaboration with Kiessling, Elmqvist, Ljungberg and Rydin. Detailed reports will be published later. In this paper only some exploratory fractionations of porcine pituitary extracts will be described. The method of Stehle (21) with some modifications introduced by Landgrebe and Mitchell (25) has been used to obtain material for further separations by electrophoresis and adsorption.

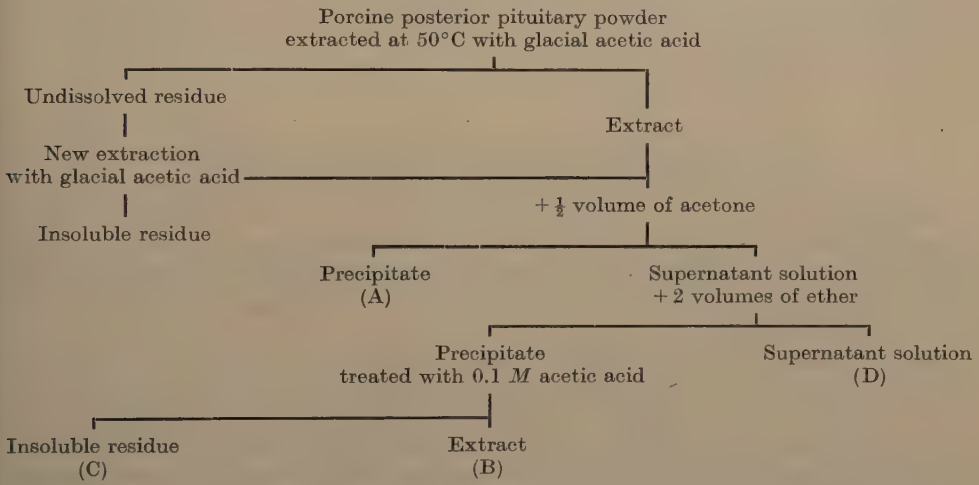
Vasopressin and oxytocin were determined by biological assay. Their distribution facilitates the evaluation of the efficiency of the fractionation methods used. As will be shown they can easily be isolated. The pure compounds will serve as guides for purification of other hormones hitherto not isolated.

Oxytocin and vasopressin, are believed to be synthesized in neurosecretory cells in the nuclei of the hypothalamus and transported within the nerve axons down through the hypophyseal stalk to the posterior lobe where they accumulate (35).

Experimental

A. *Initial steps*

The procedure described by Landgrebe and Mitchell (25) was followed except for the use of 5 times larger quantities of starting material. Therefore no description of experimental details will be given. The steps involved are summarized in the fractionation chart below (some minor details have been left out and may be found in the description of Landgrebe and Mitchell).



The dark brown preparation (D) contains amino acids, peptide material and lipids. The latter together with pigments were removed by extraction with ethyl acetate:

One volume of solution was diluted with 0.4 volumes of water and 1 volume of ethyl acetate and thoroughly shaken in a separatory funnel. The lower layer (Extract 1) was removed. The upper layer was shaken with another 0.2 volumes of water. The aqueous layer (Extract 2) was removed and the upper phase extracted again with 0.2 volumes of water (Extract 3). The extracts 1, 2 and 3 were pooled and evaporated in a rotating evaporator to approximately one third of the pooled extract volume and then lyophilized. A light brown fluffy powder was obtained: Preparation D*.

The following weight and activity distribution was obtained in one experiment:

Table 1.

Material	Dry weight (g)	Rat pressor activity USP units/mg	Avian depressor activity USP units/mg
Original powder	50	1.5	1.5
A	5.5	0.25	0.45
B	6.4	5	5
C	2.6	—	—
D*	3.5	<2	1

Preparation B which also contains the main part of the melanophore-stimulating hormones have been further fractionated by means of ionic cellulose derivatives and electrophoresis.

B. *Fractionation of Preparation B on TEAE-cellulose*

420 mg of Fraction B was dissolved in 4 ml of water and introduced into a column (1.7×11 cm) containing 3.6 g TEAE-cellulose (36) equilibrated with 0.02 *M* triethylammonium acetate. The capacity of the exchanger was calculated from the nitrogen content and was found to be 0.85 meq. per gram dry weight of the TEAE-cellulose powder.

The chromatogram was developed with 0.02 *M* triethylammonium acetate at a rate of 0.6 ml per minute. When 24 fractions, 3 ml each, had been collected, the 0.02 *M* solution was replaced by 0.1 *M* triethylammonium acetate. After another 24 fractions the bulk of the remaining material was displaced by 0.1 molar acetic acid. Absorption at 280 mμ was determined for each fraction. The resulting curve showed four peaks (Fig. 1). The material in three pooled fractions denoted Bα, Bβ and Bγ was separately lyophilized, weighed and assayed with respect to pressor and avian depressor activity. The results are compiled in Table 2.

Table 2.

Material	Fraction range in the chromatogram (Fig. 1)	Weight (mg)	Pressor activity USP units/mg	Avian depressor activity USP units/mg
Original material (B)	—	420	~ 7	~ 7
Bα	4–28	127	20	19
Bβ	29–54	32	<0.3	<1
Bγ	55–62	260	<0.3	<1

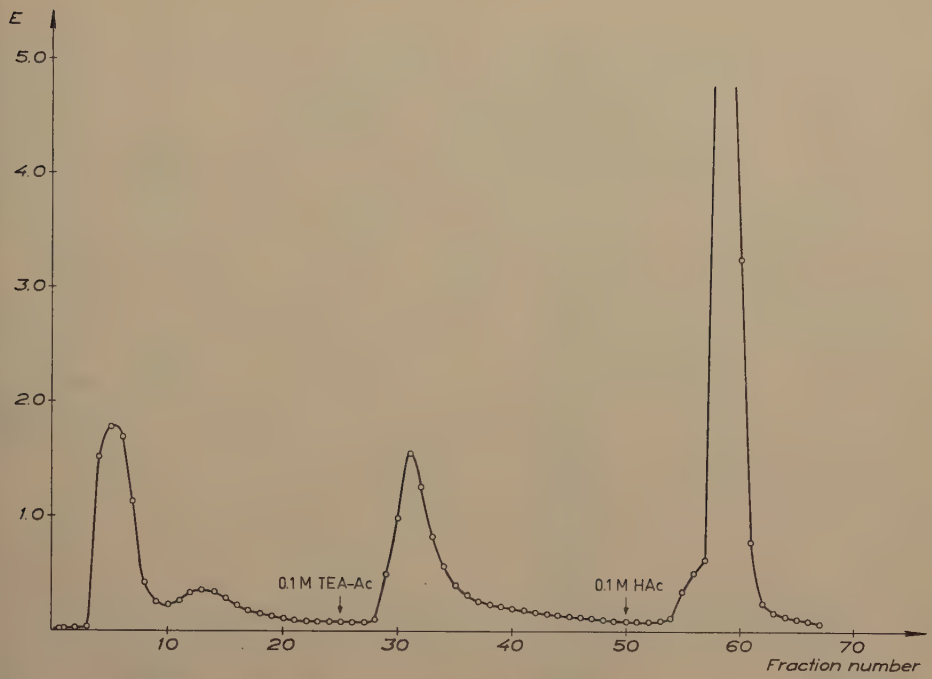


Fig. 1. Chromatographic separation of 420 mg of Preparation B on a 1.7 × 11 cm column of TEAE-cellulose. The optical density at 280 mμ is plotted versus the fraction number. Each fraction contained 3 ml of solution. The arrows indicate the number of fractions at which new displacers were placed on top of the column.

C. *Electrophoretic fractionations of Preparation Bα. Isolation of oxytocin*

Fraction Bα has been electrophoretically fractionated in 1 *M* acetic acid, in triethylamine–formic acid and pyridine–formic acid buffer systems. In the acetic acid solution the author obtained extremely good separations. All substances in the sample detected by ninhydrin and UV-absorption moved toward the cathode. At least three peptides with higher mobility than vasopressin were detected. Unfortunately large loss of activity was encountered. Only fractionations in the two other electrolyte systems will therefore be described in the present paper.

1. *Fractionation in triethylamine–formic acid buffer*

Preliminary electrophoretic runs of Fraction Bα were made on paper in a number of buffer systems made up of triethylamine and formic acid in various proportions. After a subjective judgement of differences in distribution of ninhydrin positive material obtained a mixture of 0.25 *M* formic acid adjusted with triethylamine to pH 2.35 was chosen for column electrophoresis. This buffer had a specific conductivity of 1.3×10^{-3} ohm^{-1}cm^{-1}.

The preparative electrophoresis was performed in an externally cooled column (Fig. 2), 300 cm in length and 1.5 cm diameter, of a type previously described (37, 38,

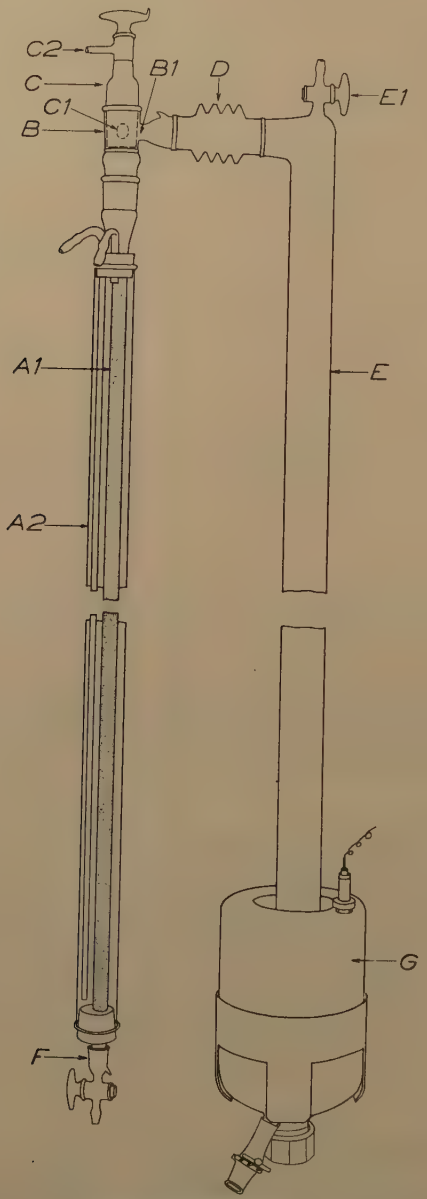


Fig. 2. A simple apparatus for column electrophoresis. The electrophoresis tube *A*1 is surrounded by a cooling jacket *A*2. *F* at the lower end of the column permits the control of the liquid flow when the mixture is introduced and displaced to the starting position in the column. At the upper end *B* and *C* connect *A*1 to *E* via the flexible, corrugated perbunan tube *D*. The lower end of *E* is immersed in the electrode vessel *G*, a converted polythene flask with the central part of the bottom removed. *E* is filled with buffer solution by suction at *E*1. Liquid contact between *A*1 and *G* is obtained by turning *C* in *B* so that the hole *C*1 coincides with the opening *B*1 in the female joint of *B*. During electrophoresis *F* is removed and the lower end of the electrophoresis tube is placed in an electrode vessel similar to *G*. After the end of electrophoresis *B*1 is closed and *C*2 connected to a buffer container placed so that a suitable flow of buffer through the column is obtained.

39). Packing of the cellulose powder ("Munktell cellulose powder") was performed according to the direction of Flodin and Kupke (40). The column was washed with two liters of buffer solution prior to use. When the buffer container was placed at the top of the column one hold-up volume (470 ml) of solution passed through the column in 9–10 hours. A top piece *B* (Fig. 2) was attached to the column and connected to the side arm of the "connecting limb" *E* by means of a corrugated perbunan tube *D* (cf. (39)). An end piece *F* with a stopcock was placed at the bottom of the column in order to make possible the regulation of flow.

160 mg of preparation Bα was dissolved in 2.5 ml of 0.25 M formic acid and 1 mg of ε-DNP-lysine was added as a marker. This solution (specific conductivity 2.0×10^{-3} $ohm^{-1}cm^{-1}$) was placed on top of the drained column and slowly forced into the column by gravity. At the moment when the last free liquid disappeared above the surface of the cellulose bed 2–3 ml of buffer solution was added and also washed into the column. All yellow material appeared now as a narrow band (approximately 2 cm wide) inside the column. The band was forced down with buffer solution to a position 7.0–12.5 cm from the top, with the stopcock at the lower end open. The flow through the column was stopped by closing the stopcock. The top piece *B* was closed by the head *C*, which has a hole *C*1 in its male joint; by turning *C* in *B*, *C*1 can be brought to coincide with the side opening *B*1 in the female joint of *B*. The lower end of *E* was placed in a large converted polythene flask *G* which served as electrode vessel. The latter was filled with 7 liters of buffer solution. Liquid connection between the column and this electrode vessel was brought about by attaching *E*1 via a rubber tubing to a water pump and raising the liquid by suction. More detailed descriptions of this operation have been given elsewhere (37, 38, 39). With the connection between *E* and the column closed the end-piece at the bottom of the column was removed. The other electrode vessel filled with buffer solution was placed under the column. Leveling of the surfaces of the solutions in the two electrode compartments was accomplished by a siphon which was thereafter removed. *C* was now turned to give open liquid connection between the electrode vessels. Cooling water of 10.5°C was circulated through the cooling jacket. The electrodes were connected to a power supply (able to yield stabilized voltage in the range 0–3500 V and direct current 0–1.0 A) with the negative electrode at the bottom of the column.

2800 volts yielded a current of 20.5 mA. After 5 hours' electrophoresis the yellow zone had sharpened (approximate position: 10.5–12.5 cm below the upper surface of the cellulose). During the following electrophoresis a slight widening of the yellow zone was observed as it moved down the column. The electrophoresis was continued for several days and the position of the yellow zone was measured each day. The total displacement of the yellow zone from the starting position was found to be proportional to the time from the start of the run. After 123 hours' total electrophoresis time the yellow band had reached a position approximately 86.5–91.5 cm below the cellulose surface. The electrophoresis was now stopped. The junction to *E* was disconnected by turning *C* and the column connected to a buffer container via *C*2. The electrode vessel below the column was removed and replaced by a fraction collector. The liquid in the column was displaced at a rate of 60 ml per hour. 5 ml fractions were collected.

From each fraction a 0.2 ml sample was taken and analysed with the ninhydrin reaction according to Moore and Stein (41). 0.1 ml samples from every other fraction were hydrolyzed in 1 ml 6 *M* HCl at 120°C for 12 hours and then subjected

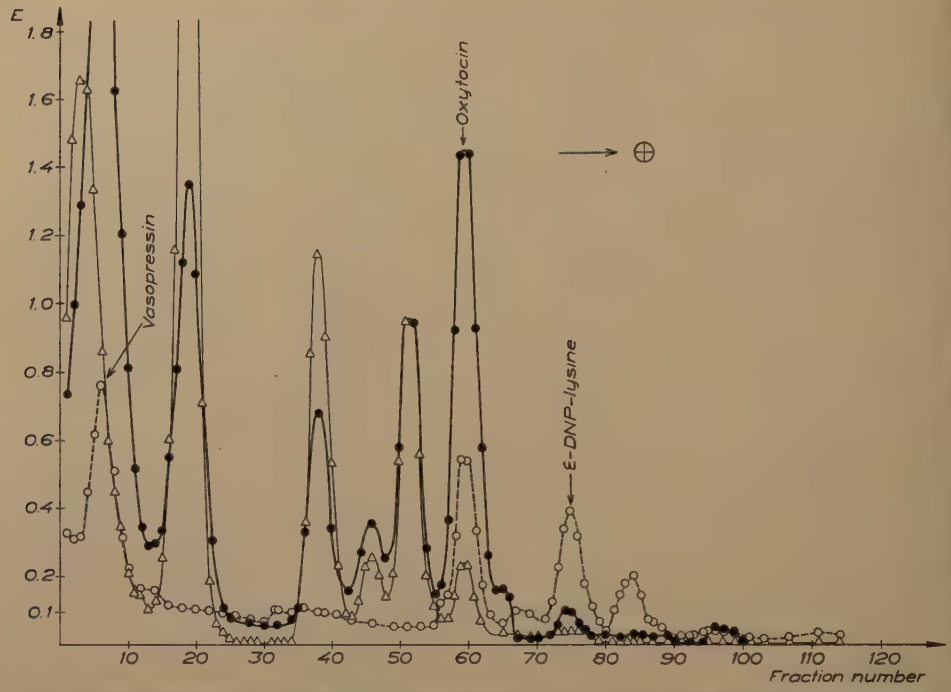


Fig. 3. Electropherogram of 160 mg of Preparation Bα. The run was made on a 1.5 × 300 cm column in a triethylamine–formic acid buffer of pH 2.35. The conditions are decribed in the text. ε-DNP-lysine was employed as a marker. The direction of current in indicated by the sign →⊕.

●–●–●–● Ninhydrin curve obtained from hydrolyzed 0.1 ml samples
△–△–△–△ Ninhydrin curve obtained from unhydrolyzed 0.1 ml samples
-○- -○- -○- UV-absorption curve (280 mμ)

Table 3.

Fraction	Volume range (see Fig. 3)	Pressor activity USP units/ml	Avian depressor activity USP units/ml
Bα1*	0– 60	ca. 50	ca. 5
Bα2	60–150	0.8	0
Bα3	150–215	0.15	0.5
Bα4	215–240	0.25	0
Bα5	240–280	0	0
Bα6*	280–320	< 0.5	ca. 65
Bα7	320–400	< 0.05	0
Bα8	400–430	0	—
Bα9	430–570	0	0

* Bα1 yielded 28 mg and Bα6 7 mg on lyophilization.

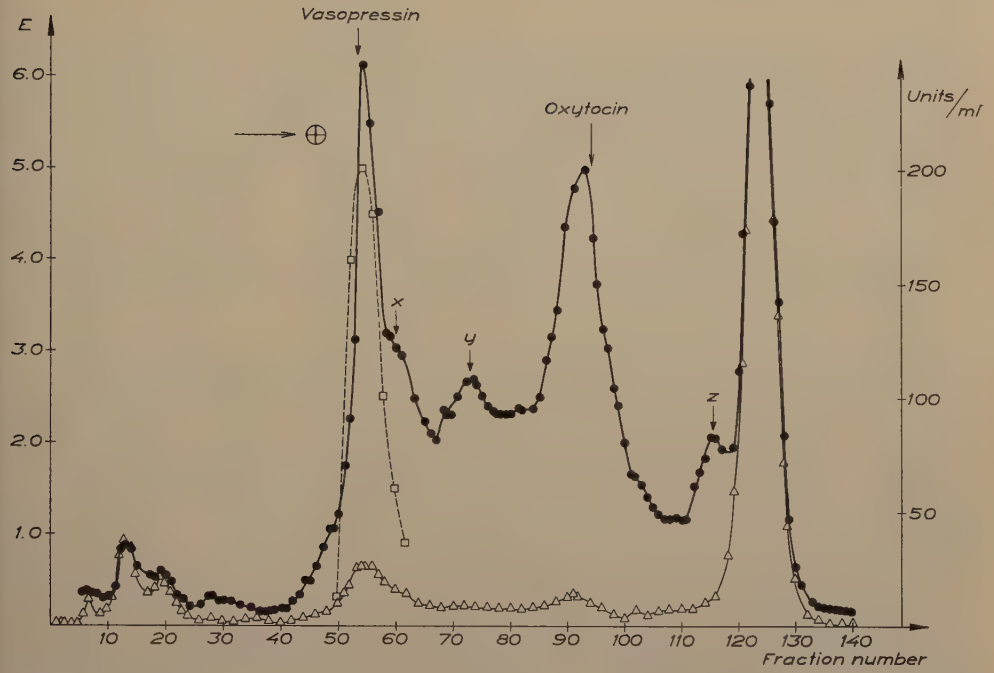


Fig. 4. Electropherogram of 460 mg of Preparation Bα obtained after 130 hours of electrophoresis at 45 mA in a 2 × 250 cm column using 0.1 *M* pyridinium formate. Fractions of 5 milliliter each were collected.

●–●–●–● Ninhydrin curve obtained from hydrolyzed 0.1 ml samples
△–△–△–△ Ninhydrin curve obtained from unhydrolyzed 0.1 ml samples
-□- -□- -□- Pressor activity curve (USP units per milliliter)

to ninhydrin analysis (v. Hofsten (42)). Absorption at 280 and at 360 mμ (the yellow zone) was measured on undiluted fractions. The diagram thus obtained is shown in Fig. 3.

The fractions were pooled according to Table 3 and the pressor (43) and avian depressor activities (44) were determined on the solution volumes indicated in the table.

3. *Fractionation in pyridine-formic acid buffer*

460 mg of Preparation Bα dissolved in 5 ml 0.1 *M* pyridinium formate was introduced in a 2×250 cm cellulose column. The electrophoresis was started after the initial zone had been displaced 15 cm below the surface of the cellulose. In order to prevent concentration changes the buffer solution was circulated between the two electrode vessels (cf. 45). After 130 hours' run at 45 mA (1175 V), the column liquid was displaced at a rate of 100 ml per hour. 5 ml fractions were collected. Aliquots of 0.1 ml each were taken for ninhydrin analysis of unhydrolyzed material. The approximate distribution of rat pressor and avian depressor activity was determined. The result is shown in Fig. 4. Fractions containing the shoulder *x* and the two maxima *y* and *z* were pooled as shown in Table 4.

Table 4.

Preparation	Fraction numbers	mg dry material
Bα-*x*	58– 65	9
Bα-*y*	65– 80	26
Bα-*z*	112–117	19

The possible biological significance of these preparations is under investigation.

D. *Fractionation of Preparation Bα-z on sulfomethyl cellulose followed by zone electrophoresis in pyridinium acetate. Isolation of β-MSH*

12 mg of Preparation Bα-*z*, dissolved in 0.02 *M* trimethylammonium aceate, was placed on a column (1.2×12 cm) of sulfomethyl cellulose (36) (0.43 meq. S/g adsorbent) in equilibrium with 0.02 *M* trimethylammonium acetate. The chromatogram was developed at a rate of 20 ml per hour with 100 ml of this buffer followed by 0.1 *M* trimethylammonium acetate. Fractions, each containing 3 ml, were collected and the absorption measured at 280 mμ. Two peaks were obtained in the chromatogram. The one corresponding to unadsorbed substance was lyophilized yielding 8 mg of dry powder. This powder was dissolved in 1 ml of 0.1

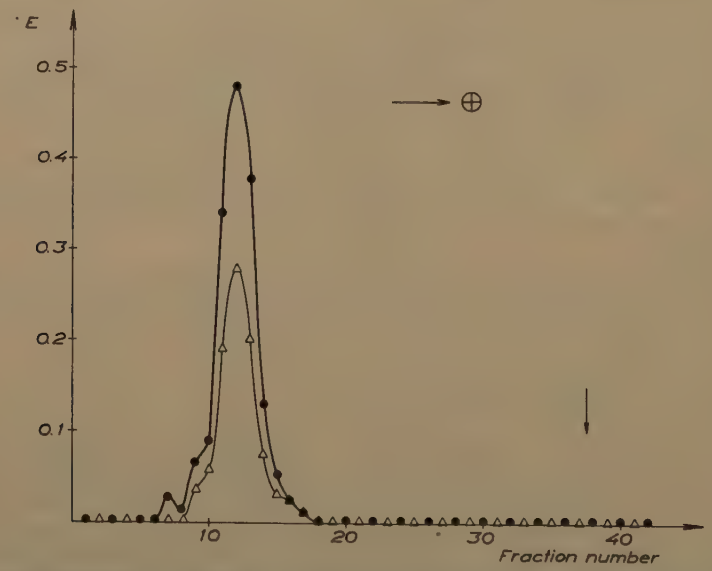


Fig. 5. Electropherogram of 8 mg of a fraction obtained from Preparation Bα-*z* by chromatography on SM-cellulose. The electrophoresis in this experiment was performed at 50 mA for 45 hours in a 1.8×52 cm column, 0.1 *M* pyridinium acetate being used. Fractions containing 2.7 ml were collected.

●–●–●–● Ninhydrin curve obtained from hydrolyzed 0.1 ml samples
△–△–△–△ Ninhydrin curve obtained from unhydrolyzed 0.5 ml samples

molar pyridinium acetate and transferred to a column for zone electrophoresis (1.2×52 cm) with 0.1 molar pyridinium acetate. The electrophoresis was started with the material containing zone 5 cm below the cellulose surface. After 45 hours' run at 50 mA the electrophoresis was stopped and the column liquid displaced at a rate of about 50 ml per hour and collected in fractions of approximately 2.7 ml each. Aliquots of 0.5 ml were taken for ninhydrin determination on unhydrolyzed and 0.1 ml for hydrolyzed material. The distribution curves obtained are shown in Fig. 5.

The second peak from the chromatogram also yielded an electrophoretically homogeneous substance but of lower mobility than β-MSH.

E. *Fractionation of Preparation B on DEAE-cellulose followed by SM-cellulose*

5 g of Preparation B in 200 ml of water was thoroughly stirred with 30 g DEAE-cellulose (0.45 meq. N/g adsorbent) in free form. After 1 hour the ion exchanger was removed on a filter and washed with 300 ml of water. The solution was filtered through a column (3.5×12 cm) containing DEAE-cellulose (0.45 meq. N/g) in free form. The column was washed with 100 ml and the washing liquid and eluate mixed and transferred to a 1.2×45 cm column of free SM-cellulose (0.43 meq. S/g). Eluate and subsequent washing solution were combined (about 700 ml together) and lyophilized yielding Preparation B1. The adsorbed material from the DEAE-cellulose was displaced by 10 per cent acetic acid yielding Preparation B2. The substance adsorbed on SM-cellulose was displaced by 80 per cent acetic acid yielding Preparation B3. The following distribution of material and activities was obtained:

Table 5.

Preparation	Weight (g)	Activity in USP units/mg	
		Avian depressor	Rat pressor
B1	0.70	2	2
B2	3.70	6	0.2
B3	0.12	7	65

Preparation B3 appeared to be contaminated by soluble material from the SM-cellulose.

F. *Isolation of vasopressin from Preparation B3*

35 mg of B3 was dissolved in 5 ml of water and displaced 50 ml below the surface of the cellulose in an electrophoresis column (2×250 cm) containing a mixture of 0.3 *M* pyridine and 0.1 *M* acetic acid as electrolyte. After electrophoresis for 90 hours with a current of 50 mA (980 V) the solution in the column was displaced and collected in 5 ml fractions. Material distribution was determined on unhydrolyzed and hydrolyzed 0.1 ml aliquots and the pressor activity determined (Fig. 6). The solutions in fractions 21, 22, 23 and 24 were pooled and lyophilized. 9.6 mg dry substance was recovered.

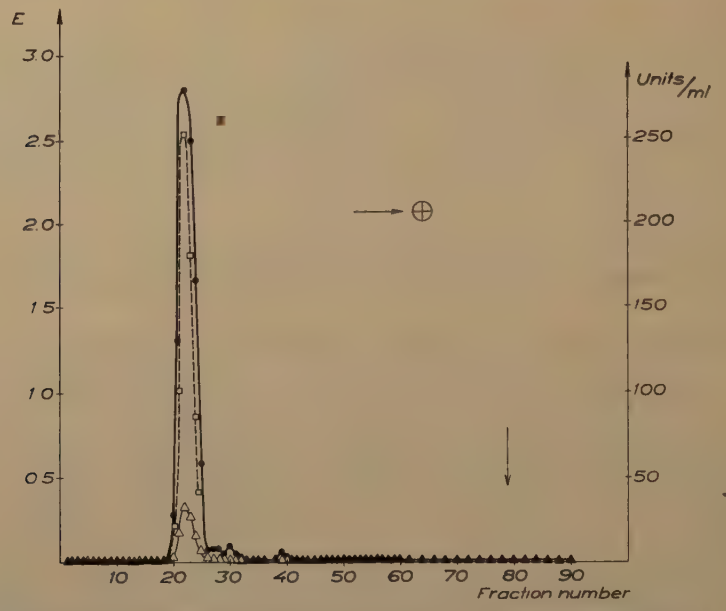


Fig. 6. Electrophoresis of Preparation B3 in a solution of 0.3 *M* pyridine and 0.1 *M* acetic acid as electrolyte. The starting position of the zone is indicated by the arrow. The run was performed in a 2 × 250 cm column at 50 mA. The electrophoresis was run for 90 hours and fractions of 5 ml were collected.

●–●–●–● Ninhydrin curve obtained from hydrolyzed 0.1 ml samples
△–△–△–△ Ninhydrin curve obtained from unhydrolyzed 0.1 ml samples
-□- -□- -□- Pressor activity curve (USP units per ml)

Discussion

If a scheme for purification of oxytocin and vasopressin could be worked out on an intermediate scale (10–100 g acetone dried posterior lobe powder) it should, in principle, be possible to use the same procedure for industrial production of these hormones in pure form. The importance of manufacturing "physiologically pure" hormones for scientific and clinical use is obvious. The same sequence of purification steps should also yield pure hormones if applied to small quantities of the powder (one gram or less). It should thus be possible to isolate hormones from rare sources, e.g. human pituitaries. In view of the chemical differences already found between beef and hog vasopressins it might be possible to make important contributions to comparative vertebrate biochemistry. Moreover, efficient fractionation procedures for vasopressin and oxytocin would also be useful for isolation of other peptide hormones present in the posterior lobe of the pituitary.

The present investigation is an extension of fractionation along lines initiated by Stehle (21) and followed by Landgrebe and Mitchell (25) and others (27, 28, 29, 33) for concentration of the melanophore expanding principle. An effective breakdown of labile complexes of the active peptides and inert substances is accomplished by the acetic acid extraction. The subsequent precipitation steps eliminate the greater part of the protein (Precipitate A) as well as amino acids and lipids (Solution D), thus leaving a fraction, B, enriched in peptides. Preparation

B contains 10–15 per cent of the original powder but the greater part of the vasopressor and avian depressor activity (see Table 1) and also the melanophore-stimulating hormone (α and β MSH), and the mammary gland stimulating factor of Bradley (46). It is apparently a good starting material for attempted isolation of these hormones.

The purification procedure may be extended efficiently by means of ion exchangers and zone electrophoresis along different lines. One possibility would be to make a preliminary electrophoresis at an intermediate pH e.g. in a pyridine-acetic acid buffer system. It should thus be possible to remove inactive acidic, neutral and extremely fast moving basic substances and obtain a fraction of moderately basic compounds with all the known hormones of the posterior pituitary. The composition of this fraction should closely resemble the pooled fraction 45–100 in the experiment referred to in Fig. 5. Further purification could be achieved with a combination of ion exchange chromatography and prolonged electrophoresis. This procedure which appears to be a promising approach is under investigation.

Preliminary fractionation by ion exchangers followed by zone electrophoresis may be used to advantage. If it is desired, in preparative work (i.e. with heavy loads) that the hormones should be adsorbed the conditions for adsorption and desorption must be carefully controlled. A more reliable method for concentration of the hormones in the posterior pituitary consists in adsorbing most of the inactive material under conditions where the hormones remain unadsorbed. Because of the basic properties of the hormones cationic ion exchangers are less favourable for this purpose.

Fractionation of Preparation B on anionic exchangers such as TEAE- or DEAE-cellulose is particularly satisfactory because oxytocin, vasopressin, MSH, and possibly also other hormones can be enriched together in a fraction, Preparation Bα (Table 2, Fig. 1), consisting of compounds which are not or only to a slight extent adsorbed. Bα contains the major portion of oxytocin and vasopressin but only about 5 per cent of the material of the original posterior lobe powder. The fact that the hormones are grouped together makes possible the isolation of vasopressin and oxytocin and presumably also the other hormones in high yield without expanding the fractionation scheme seriously.

It is known that low molecular weight substances such as neutral amino acids can be very efficiently fractionated by electrophoresis in 1 *M* acetic acid (38). Moreover, electrolyte solutions of low pH readily dissolve large quantities of Bα, thus making it possible to introduce much material in a column as a narrow starting zone. Electrophoresis in acidic buffers in this pH range would therefore be expected to give high resolution of complex mixtures such as the hormone concentrate Bα. Fig. 3 shows convincingly that this expectation is fulfilled when formic acid-triethylamine buffer is employed. The fractions 280–320 in this experiment designated Bα6, contain apparently pure oxytocin assaying 400–450 USP units per milligram of dry substance. The constant ratio of the optical densities of ninhydrin color before and after hydrolysis over the peak in this region indicates that only a single polypeptide is present in significant amounts in this region of the electropherogram. The shape of the UV-curve with a maximum coinciding with the ninhydrin peaks yields further evidence for the purity of the peptide. Thus, oxytocin has been isolated in surprisingly few steps and in a high yield. Pure vasopressin, however, is not easily obtained when buffers of low pH are employed. Most basic peptides move with high and not markedly different mobilities. Prep-

aration Bα 1 consists of about 40 per cent vasopressin. The ninhydrin peaks corresponding to Preparation Bα 2 (maximum in fraction 19) and Preparation Bα 3 (maximum in fraction 38) are chiefly due to neutral amino acids. Preparations Bα 4 and Bα 5 appear to consist of almost pure peptides without pressor or depressor activity.

Very different distribution patterns are obtained when electrophoresis is performed in a buffer of higher pH, e.g. in pyridine–formic acid or pyridine–acetic acid systems. The neutral amino acids are grouped together with uncharged substances. Fig. 4 shows a separation obtained in 0.1 *M* pyridinium formate. Vasopressin and oxytocin are completely separated from each other but neither is obtained in pure form. The shoulders in the ninhydrin curves around fraction 50, indicated by *x* in Fig. 4, and the two maxima indicated by *y* and *z*, are due to polypeptides. *z* consists of two main components one of which is β-MSH. The presence of β-MSH is supported by the following facts. The material in the tubes 11, 12 and 13 of the electropherogram shown in Fig. 5 has the same amino acid composition as β-MSH, as judged from paper chromatograms of hydrolysates. The two compounds cannot be electrophoretically distinguished. *x* or *y* may be chiefly α-MSH. Further attempts are being made to isolate the main components of the Preparations *x* and *y*. The three components moving far ahead of vasopressin appear to be lysine and possibly two amines. A very fast-moving compound had left the electrophoresis column in the experiment described.

When B was treated with DEAE-cellulose in free form most of the oxytocin was adsorbed. The reason for this unexpected behaviour is not fully understood. It is unlikely that minute amounts of carboxyl groups should cause the adsorption, since the affinity of a carboxylic ion exchanger for vasopressin should presumably be greater than that for oxytocin. Vasopressin was removed from the unadsorbed fraction by adsorption on SM-cellulose. Elution with 80 per cent acetic acid yielded a vasopressin concentrate, B3, consisting chiefly of vasopressin, an extremely basic substance and contaminants from the ion exchanger itself. The yield of vasopressin was fairly low, apparently because of incomplete elution.

In the experiment referred to in Fig. 6 pure vasopressin was obtained from B3 after a single electrophoretic run in pyridine–acetic acid buffer of pH 5.65, the same buffer solution that has recently been used by Ward and du Vigneaud for partial purification (47). Thus porcine, vasopressin, "lysine vasopressin", has been isolated. The preparation was extensively assayed and found to possess an activity of 280 units per mg, thus of the same order or slightly higher than that reported by Ward and du Vigneaud.

SUMMARY

Application of zone electrophoresis and chromatography on cellulose ion exchangers to the purification of posterior pituitary extracts have resulted in isolation of porcine oxytocin and vasopressin as well as a melanocyte stimulating hormone β-MSH, previously isolated by the author and his collaborators by another method of fractionation. The experiments reported here make it apparent that purification methods based upon a combination of chromatography on cellulose ion exchangers and zone electrophoresis using cellulose columns are more direct and less complicated than other methods hitherto used. The methods employed by the author may presumably be very useful also for purification of other natural products such as enzymes, antibodies, nucleotides, nucleic acids etc.

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Inhibition of yeast invertase (saccharase) by metal ions

IV. Inhibition by Ag^+ and the complexing of Ag^+ by certain sulphur-containing compounds

By Karl Myrbäck and Ebba Willstaedt

With 5 figures in text

Yeast invertase is inhibited by extremely low concentrations of silver ions but the nature of the silver-binding groups in the enzyme molecule is not known. Assuming that the enzyme is a simple protein without any special prosthetic group it is concluded that the silver-binding groups belong to known amino acids. In a preceding paper (1) it was shown that of sulphur-free amino acids only histidine has a silver-binding capacity that could possibly explain the inhibition of the enzyme by silver ions, especially as the binding of Ag^+ by histidine shows the same strong dependence on pH as the enzyme inhibition by Ag^+. The Ag–histidine complex, however, is considerably more strongly dissociated than is the enzyme–Ag complex.

When seeking for groups in an enzyme protein that could explain the strong binding of silver and certain other heavy metal ions one might be tempted to assume, more or less arbitrarily, that the sulphur-containing amino acids, and especially such with free HS groups, are responsible. However, the inhibition of yeast invertase by Ag^+ shows several peculiarities which make such an assumption appear somewhat doubtful:

(i) The silver inhibition is strongly dependent on pH (2). The relative activity (referred to the activity of the non-inhibited enzyme at the pH optimum around pH 4.5) is given approximately by the following formula

$$\text{Relative activity} = \frac{1}{1+\frac{K_A}{[H^+]}\left[1+\frac{[Ag^+_{tot}]-[AgE]}{K_{Ag}}\right]},$$

where $K_A \sim 10^{-6.7}$. $[Ag^+_{tot}]$ is the total concentration of silver ion added, and [AgE] is the concentration of the enzyme-silver compound. (K_A is the dissociation constant of the "acidic group" assumed, originally by Michaelis (3), to be responsible for the alkaline branch of the activity-pH curve of the enzyme). At higher Ag^+ concentrations where [AgE] may be regarded as negligible compared to $[Ag^+_{tot}]$, the formula for a certain value of $[Ag^+_{tot}]$ simplifies to

$$\text{Relative activity} = \frac{1}{1+\text{const.}\,\frac{K_A}{[H^+]}}$$

showing that the "inhibition-pH curves" arise approximately by a displacement of the alkaline branch of the activity-pH curve towards the acid side (2).

(ii) K_{Ag}, the dissociation constant of the enzyme–Ag compound, should have a value 10^{-7}–10^{-8} (1, 2).

(iii). The inhibition of invertase by several other cations as Cu^{++}, Zn^{++}, Cd^{++}, Pb^{++}, UO_2^{++} (2, 4, 1) depends on pH in the same way as does the inhibition by Ag^+.

In the following experiments the silver-binding capacity of certain sulphur-containing substances was determined according to two different methods (5).

All reagents used were p.a. grade. Redistilled water was used throughout. All solutions were freed from air as far as possible. The enzyme preparations used were partly purified from autolysates of baker's yeast.

(*a*) *Enzymatic experiments.*—Since the inactivation of invertase by Ag^+ is quite reversible and independent of substrate (saccharose) concentration, the silver-binding capacity of a substance X can be measured by determination of the relative activity of the enzyme in the presence of known concentrations of Ag^+ and X, provided that X alone has no measurable action on the enzyme; parallel experiments with the same concentration of Ag^+ without addition of X.

Unless otherwise stated, all experiments were carried out in sodium acetate–acetic acid buffer (0.01 *N* sodium acetate). The buffer concentration is so low (4) that the complexing of Ag^+ by the acetate ion does not introduce any serious errors. pH was determined by means of the glass electrode in every reaction mixture.

(*b*) *Electrometric measurements.*—The Ag^+ concentration in mixtures of $AgNO_3$ and substance X was measured with Ag electrodes against $AgNO_3$ of the same concentration. Both electrode vessels contained sodium acetate–acetic acid buffer of the same concentration (0.05 *N* sodium acetate). The calculated values of $[Ag^+]$ are only approximately correct since the ionic strength was not quite constant.

Experiments with reduced glutathione (GSH)

(*a*) *Enzymatic experiments*—The relative activity (RA) of the enzyme at different concentrations of Ag without glutathione was determined (Table 1; curve 1 in Fig. 1). The table shows that the values of [Ag] ($\alpha/(1-\alpha)$) are very far from constant. This

Table 1. Inactivation by Ag^+ at pH 5.3.

$[Ag_{tot}] \times 10^6$	Relative activity α	$[Ag]\frac{1}{1-\alpha} \times 10^6$
1	95.5	21
2	88.5	15
3	61.8	4.8
4	34.6	2.1
5	19.1	1.2
7	10.0	0.8
10	3.2	0.3

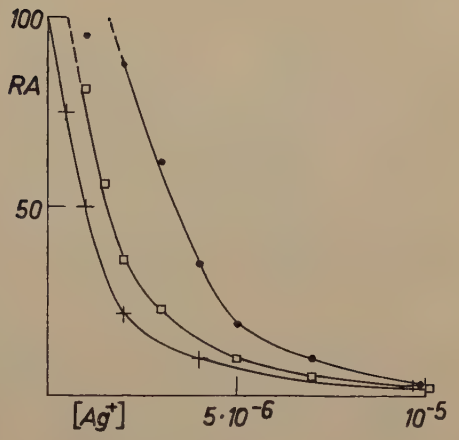

Fig. 1. Dependence of the relative activity (RA) on Ag^+ concentration. Different enzyme preparations, cf. text. Curve 1 •——•; curve 2 +——+; curve 3 □——□.

is chiefly due to the fact that the amount of Ag^+ bound by the enzyme (and by impurities in the enzyme preparation) are not at all negligible compared to $[Ag^+_{tot}]$. As pointed out before, the shape of curve 1 in Fig. 1 shows the presence in the enzyme preparation of some impurity which binds Ag much more strongly than the enzyme. This is evident from a comparison of curve 1 with the corresponding curve (curve 2 in Fig. 1) obtained with a highly purified invertase preparation (2).

Table 2 shows the relative activity of the enzyme in presence of 5×10^{-6} *N* Ag^+ and different concentrations of glutathione. Preliminary experiments allowed the conclusion that the complexing of Ag^+ by glutathione is instantaneous; in the experiments of Table 2 the enzyme (1 ml) was added 1 min. after mixing of Ag^+, buffer, substrate and glutathione (99 ml).

Table 2. Action of reduced glutathione at pH 5.3.

Experimental values		Calculated values	
$[GSH] \times 10^6$	Relative activity	$[Ag^+_{corr}] \times 10^6$	Relative activity
—	22.7		
0.05	24.1		
0.5	38.4	4	35
1	59.1	3	62
1.5	79.5	2	88
2	95.4	1	95
5	100	0	100
100	100	0	100

It appears from Table 2 that glutathione binds Ag^+ very strongly; complete protection of the enzyme is obtained at very low glutathione concentrations. Fig. 2 shows that at a total Ag concentration of 5×10^{-6} full activity of the enzyme, i.e. complete removal of all free Ag^+ ions, is reached at a concentration of glutathione slightly above 2×10^{-6} *M*. It must be concluded at the prevailing conditions one molecule of glutathione binds two Ag ions.

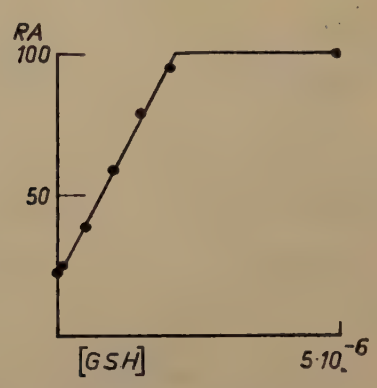

Fig. 2. Action of glutathione on the inhibition by 5×10^{-6} *N* Ag^+.

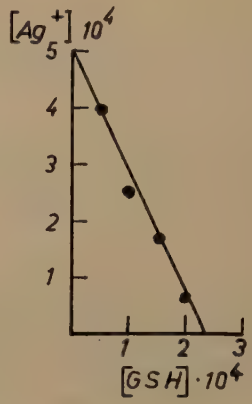

Fig. 3. Concentration of free Ag^+ ion in glutathione solutions.

Electrometric experiments.—Complexing of the Ag ion by glutathione was tested in experiments with electrometric determination of Ag^+ concentration (Table 3).

The electometric determinations show conclusively that two Ag^+ ions are bound by one glutathione molecule, i.e. the determinations support strongly the conclusion arrived at in the enzymatic experiments. The straight line in Fig. 3 must mean that the dissociation constant of the Ag–glutathione complex is extremely low.

Some electrometric determinations were carried out at different pH's (Table 3). The dependence, if any, of the complex formation on pH is slight and not at all comparable to the strong dependence of the enzyme inhibition on pH.

If we assume that in the experiments of Table 2 the glutathione present binds two Ag^+ per molecule, we may calculate the concentration of Ag^+, active in the enzyme inhibition, $[Ag^+_{corr}]$, i.e. the sum of free Ag^+ and Ag bound to enzyme and impurities in the enzyme preparation. From curve 1 of Fig. 1 the relative activities corresponding to the various values of $[Ag^+_{corr}]$ can be estimated. These values agree fairly well with the experimental values (Table 2).

Table 3. Complexing of Ag^+ by glutathione.

pH 5.3		Glutathione = 1.5×10^{-4} *M*	
$[GSH] \times 10^4$	$[Ag^+] \times 10^4$	pH	$[Ag^+] \times 10^{-4}$
0	5	4.5	1.7
0.5	4.0	5.3	1.7
1	2.5	6.0	1.4
1.5	1.7	6.7	1.4
2	0.65		

Experiments with thioglycolic acid

Enzymatic experiments.—The relative activity of the enzyme at different Ag^+ concentrations without thioglycolic acid is illustrated by curve 3 in Fig. 1. The protecting action of thioglycolic acid on the enzyme against silver inactivation is shown in Table 4 and Fig. 4.

It appears that thioglycolic acid has a very strong protective action. If the amounts of Ag^+ removed from the enzyme–Ag equilibrium are calculated by comparing the activity values of Table 4 and curve 3 of Fig. 1, it seems probable that in the enzyme

Table 4. Protecting action of thioglycolic acid (TGA) at pH 5.3.

$[Ag_{tot}]$	$[TGA] \times 10^6$	Relative activity
5×10^{-6}	0	10.0
	1	14.7
	2	39.2
	3	93.8; 93.8
	5	100; 100
10^{-5}	0	2.7
	3	7.7; 11.0
	4	13.6
	5	32.0
	6	76.5
	10	100
2×10^{-5}	0	1.8
	8	9.9
	10	25.7
	11	55.2
	12	95.0
	15	100

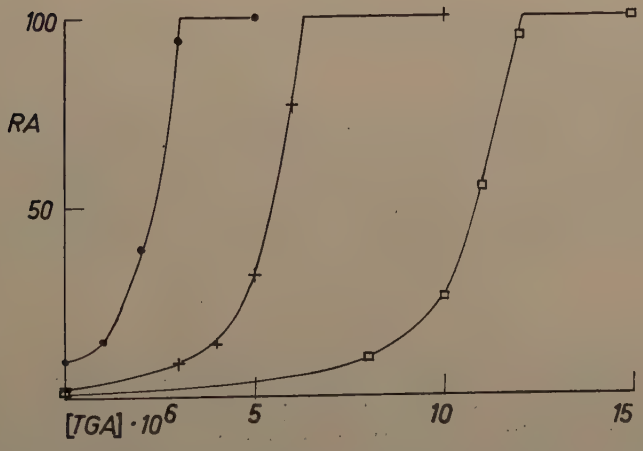

Fig. 4. Relative activity (RA) at three concentrations of Ag^+_{tot} and various concentrations of thioglycolic acid (TGA). ●, 5×10^{-6} *N* Ag^+; +, 10^{-5} *N* Ag^+; □, 2×10^{-5} Ag^+.

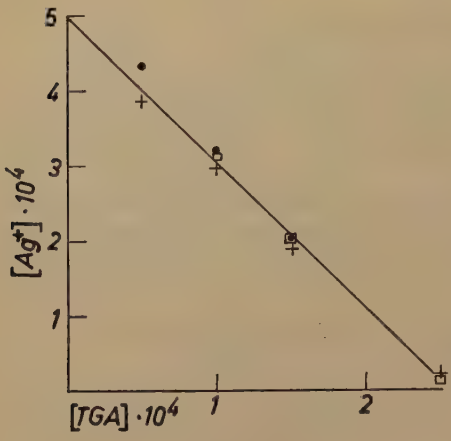

Fig. 5. Concentration of free Ag^+ ion in thioglycolic acid solutions. • pH 4.5; + pH 5.3; □ pH 6.0.

Table 5. Complexing of Ag^+ by thioglycolic acid.

pH	[TGA] × 10^4	[Ag^+] × 10^4
4.5	0	5
	0.5	4.35
	1.0	3.21
	1.5	2.02
	2.5	0.22
5.3	0.5	3.85
	1.0	2.96
	1.5	1.87
	2.5	0.22
6.0	1.0	3.15
	1.5	2.02
	2.5	0.14

experiments one molecule of thioglycolic acid has bound 1.5–2 Ag^+. This is more clearly seen from Fig. 4.

Electrometric experiments on the Ag-bindning capacity of thioglycolic acid were carried out, Table 5. This table shows that one molecule of thioglycolic acid binds two Ag ions. It is also seen from the table, and more easily from Fig. 5, that there is no dependence of the complex formation of pH in the range investigated. Finally, the dissociation of the complex is extremely small (straight line in Fig. 5).

Experiments with cysteine

Enzymatic experiments.—The relative activity of the enzyme in presence of 10^{-5} *N* Ag^+ is ~ 3. If cysteine was added to a concentration of 10^{-5} *M*, the relative activity was regularly found to be 100, which means that these mixtures do not contain measurable amounts of free Ag^+ ions. At a cysteine concentration of 5×10^{-6} the relative activity was about 50. From curve 3 in Fig. 1 it appears that this RA corresponds to $[Ag^+_{tot}] \sim 1.5 \times 10^{-6}$. This would mean that 5×10^{-6} moles of cysteine

have bound 8.5×10^{-6} Ag^+ ions. However, the experiments with these extremely diluted cysteine solutions were not very well reproducible, probably because of the rapid oxidation of cysteine by air.

Electrometric experiments.—Also in these experiments the reproducibility of the measurements of Ag^+ concentration was rather doubtful. A few measurements are shown in Table 6. It seems permissible to assume that one mole of cysteine, like the other SH compounds investigated, binds 2 Ag^+ ions. The complexing action of cysteine does not vary with pH in the range 4.5–6.7.

Table 6. Complexing of Ag^+ by cysteine.

pH	[Cysteine] $\times 10^{-4}$	[Ag^+] $\times 10^{-4}$
4.5	0	5.00
	0.5	4.08
	1.0	3.07; 3.24
	1.5	2.72
	2.0	1.65; 2.00
	2.5	1.07
	3.5	0.31
5.3	1.5	2.36
5.95	1.5	2.36
6.7	1.5	2.45

Comparing the results concerning the Ag-binding capacity of the HS compounds investigated and that of invertase, the following facts should be noted:

(*a*) The HS compounds investigated bind Ag^+ more strongly than the enzyme. The dissociation constants of the Ag-mercaptides are, at least in most cases, unmeasurably small, whereas the Ag-enzyme compound has a dissociation constant probably not lower than 10^{-8}.

(*b*) The Ag-binding capacity of the HS compounds investigated is independent of pH in the range pH 4.5–6, whereas that of invertase varies very strongly. The apparent dissociation constant of the enzyme-Ag compound varies in the ratio 1:10 if pH is altered from 6 to 5.

The experiments make it appear rather improbable that the complex formation of the enzyme with silver ions (and certain other metal ions) is mediated by free HS groups in the enzyme molecule.

It may be added that yeast invertase is rather insensitive to iodoacetate (5). When incubated with 10^{-3} *M* iodoacetate at pH 5.3 for 30 min. at 30° the enzyme suffered no inactivation, and in 10^{-2} *M* iodoacetate the activity decreased by only about 10%.

Experiments with methionine

Enzymatic experiments.—The relative activity of the enzyme at various Ag^+ concentrations and various pH's was determined (Table 7). The strong dependence of the activity on pH is evident. Experiments at $[Ag^+] = 5 \times 10^{-6}$ and different concentrations of methionine were carried out at four different pH values (Table 8).

Table 7. Inhibition by Ag^+ in absence of methionine.

$[Ag^+_{tot}] \times 10^6$	pH	Relative activity
0	4.5	100
1	4.8	93.8
	5.3	69.9
	5.7	33.8
2	4.5	91.9
	4.8	73.5
	5.3	31.3
	5.7	11.0
5	4.5	60.7
	4.8	37.5
	5.3	11.1
	5.7	2.6

Table 8. Inhibition by Ag^+ in presence of methionine; $[Ag^+_{tot}] = 5 \times 10^{-6}$.

pH	[Methionine] $\times 10^3$	Incubation of Ag^+ and methionine, min.	Relative activity
4.5	0	—	60.7
	1	1	87.5
	1	30	87.8
	2	1	95.6
4.8	0	—	37.5
	1	1	80.1
	2	1	86.5
5.3	0	—	11.0
	1	1	39.7
	1	30	39.7
	2	1	62.5
5.7	0	—	2.6
	1	1	11.1
	2	1	28.7

A comparison of Tables 7 and 8 shows that methionine has a considerable protecting action. However, this action is found only at relatively high methionine concentrations ($\sim 10^{-3}$ *M*) and, consequently, not comparable to that exerted by the HS compounds which have the same action in concentrations $\sim 10^{-6}$. From the tables it can be seen that at 10^{-3} *M* methionine and $[Ag^+_{tot}] = 5 \times 10^{-6}$ the concentration of Ag, active in the enzyme inhibition, is lowered by more than 50%. Supposing that one mole of methionine binds one silver ion, the dissociation constant of the Ag–methionine complex would be 10^{-3}–10^{-4}.

Electrometric determination of the Ag-binding capacity of methionine were carried out (Table 9). Under the assumption that one molecule of methionine binds one Ag ion a dissociation constant

$$K = \frac{[Ag^+_{free}]\,[Meth_{free}]}{[Ag_{bound}]}$$

was calculated. Table 9 shows that the *K*-values are tolerably constant. There are no variations of *K* with either methionine concentration or with pH. Thus, comparing

Table 9. Complexing of Ag^+ by methionine ("Meth").

pH	$[Meth_{tot}] \times 10^3$	$[Ag^+] \times 10^4$	$[Meth_{free}] \times 10^3$	$K \times 10^4$
4.50	0	5.00	0	—
	0.25	3.83	0.133	4.36
	0.5	2.65	0.265	2.99
	1.0	1.65	0.665	3.27
	2.5	0.698	2.07	3.36
	5.0	0.366	4.54	3.58
5.70	0.25	3.68	0.118	3.29
	0.5	2.96	0.296	4.28
	1.0	1.83	0.683	3.94
	2.5	0.725	2.07	3.52
5.95	0.25	3.70	0.120	3.42
	0.5	2.96	0.295	4.28
	1.0	1.56	0.656	2.97
	2.5	0.753	2.07	3.67
	5.0	0.356	4.54	3.48

Mean value $K = 3.60 \times 10^{-4}$

the binding of Ag^+ by methionine and by the enzyme, we find striking differences. The complexing of Ag^+ by methionine is independent of pH, whereas the inhibition of the enzyme, as mentioned before, varies strongly in the same pH range. Further, the dissociation constant of the methionine-Ag compound is about 3.6×10^{-4} but that of the enzyme-Ag compound 10^{-8}–10^{-7}.

SUMMARY

The inhibition of yeast invertase by Ag^+ ions has been compared to the complexing of Ag^+ by HS compounds (cysteine, glutathione, thioglycolic acid) and by methionine. The complexing of Ag^+ by the enzyme is strongly dependent on pH, that of the sulphur-containing substances mentioned is not. The dissociation constant of the enzyme-Ag compound is about 10^{-8}, that of the methionine-Ag compound 3.6×10^{-4} of that of the HS compounds, e.g. glutathione, is unmeasurably small. The results do not support an assumption that the silver inhibition of the enzyme is due to a reaction of the Ag ion with free HS groups or methionine groups in the enzyme.

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6-Benzylmercaptomethyl-3(2H)-pyridazinone

By Göran Claeson

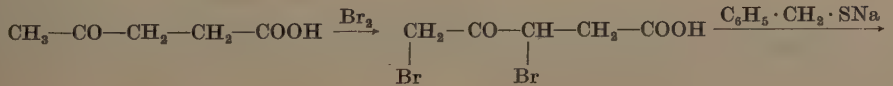
With 2 figures in the text

At this institute experiments are in progress to prepare homologues of 6-thioctic acid (1). In one of these experiments levulinic acid was chosen as starting material. This acid has been brominated previously with two moles of bromine giving a dibromo acid. The structure of this substance has also been proved to be 3,5-dibromolevulinic acid, i.e. the bromine atoms are symmetrically substituted around the carbonyl group (2, 3).

The present author has carried out the bromination in acetic acid obtaining a good yield (88%) of 3,5-dibromolevulinic acid. Bromination in ether according to Wolff (4) was also tried, and in this medium the reaction proceeded faster but the dibromo acid obtained was not quite pure.

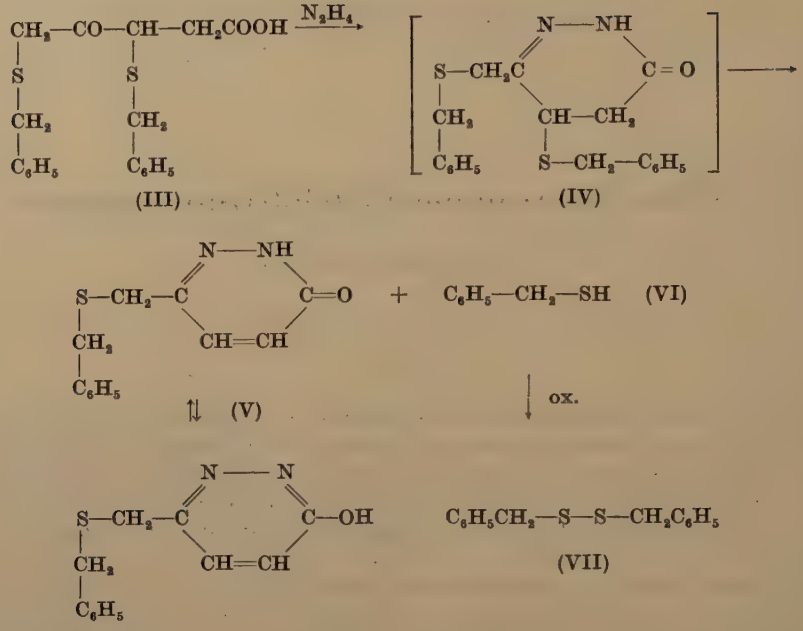
The sodium salt of 3,5-dibromolevulinic acid was treated with sodium benzylmercaptide in water solution. From the water solution two different compounds could be separated. A molecular compound (1:1) of 2,5-dibenzylmercaptolevulinic acid and its sodium salt precipitated from neutral or weakly acid solution, but at lower pH the free acid was obtained as a precipitate. Both of the compounds could be recrystallized and were obtained pure with melting points 153° and 116°.

Several attempts to reduce the carbonyl group of 3,5-dibenzylmercaptolevulinic acid have been carried out. A Clemmensen reduction gave decomposition products, which were not determined. A Wolff–Kishner reduction with hydrazine hydrate gave two different neutral reaction products, one containing sulfur and the other both sulfur and nitrogen. The first substance was proved to be dibenzyl disulfide, and the second which had amphoteric properties, soluble in both acids and bases, was assumed to be 6-benzylmercaptomethyl-3(2H)-pyridazinone[1] (V). Chemical analyses and the infrared spectrum confirmed this. The reaction with hydrazine hydrate possibly passes through the 4,5-dihydro-3(2H)-pyridazinone-derivate[1] (IV), which then gives off benzylmercaptan. A double bond is thereby formed and the heterocyclic compound is stabilized by resonance of the aromatic ring structure. The dibenzyl disulfide was obtained from benzylmercaptan (VI), which in the separation procedure was oxidized by air to disulfide.



(I) (II)

[1] Nomenclature according to *Chemical Abstracts*.



The IR-spectrum of the compound with the assumed structure (V) is shown in Fig. 1. All amides show a strong carbonyl absorption band near 6.05 μ when examined in the solid state. This absorption is termed the amide I band (5). In Fig. 1 this absorption corresponds to the peak at 6.03 μ. In spectra from cyclic lactams the amide II band is absent (6), and the strong absorption at 6.25 μ must correspond to the conjugated C=N vibration. The non-conjugated C=N absorption usually occurs near 6.1 μ, and conjugation shifts the band to higher wave-lengths (7). However, no absorption band due to the C=C vibrations could be found in the expected vicinity of 6.1 μ (8). Now it is probable that the strong amide I band overlaps the weaker C=C vibration. The frequency of the amide I band is, however, markedly affected by hydrogen bonding effects, so that considerable shifts can occur on passing from the solid state to solution. Fig. 2 shows the interesting part of the spectrum when the substance is dissolved in chloroform. The expected frequency shift from 6.03 μ to 5.95 μ is observed, the C=N band frequency is situated in the same place, but in addition a new peak appears at 6.02 μ, which corresponds to the absorption of the C=C in formula (V).

The same reaction products (V) and (VI) were obtained when the reduction was performed in diethyleneglycol at 180° according to Huang-Minlon (9), and when the keto-acid was warmed on a steam-bath with hydrazine hydrate. If usual levulinic acid or its ethyl-ester is warmed on a steam-bath with hydrazine hydrate 6-methyl-4,5-dihydro-3(2H)-pyridazinone is formed (10, 11). The hydrate of this compound loses nitrogen and is transformed into valeric acid when heated with alkali (12). On the other hand the compound (V) is not affected under the same conditions, and with more violent heating it is destroyed.

The substance (V) was tested against certain bacteria and fungi, but no inhibition of growth was observed.

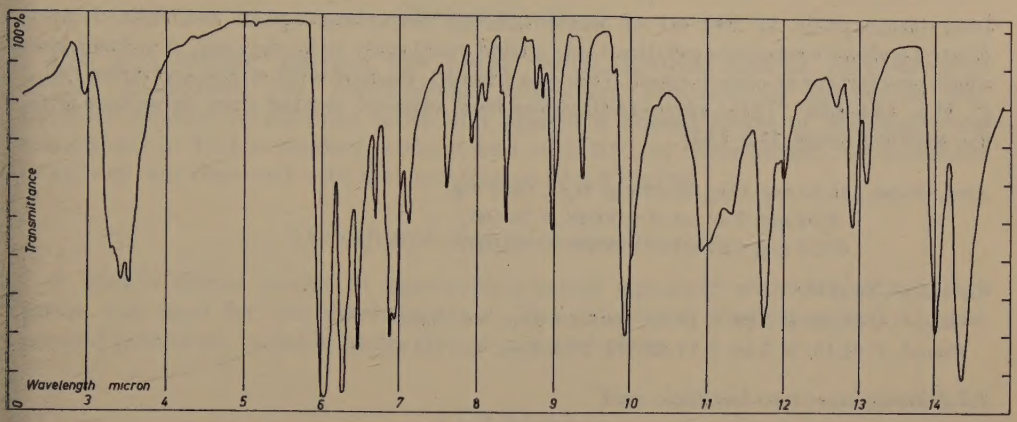


Fig. 1. IR absorption curve of 6-benzylmercaptomethyl-3(2H)-pyridazinone. KBr phase.

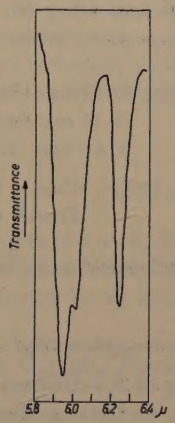


Fig. 2. IR absorption curve of 6-benzylmercaptomethyl-3(2H)-pyridazinone in chloroform.

Experimental

3,5-Dibromolevulinic acid

58 g of levulinic acid (0.5 moles) was dissolved in 50 ml of acetic acid, 150 ml of water and 2 ml of konc. hydrobromic acid. 160 g of bromine (1 mole) were slowly dropped into the solution with mechanical stirring and heating with electric bulb. The bromination required six hours. After standing overnight about 50 g of white crystals had separated, which were filtered off and washed with water. The solution was left to evaporate on a steam-bath after which further crystals could be separated. In total 120 g (88%), m.p. 108–111°. One recrystallisation from water yielded white crystals, m.p. 113–114°.

Molecular compound (1 : 1) between 3,5-dibenzylmercapto-levulinic acid and its sodium salt

27.4 g of 3,5-dibromolevulinic acid (0.1 mole) was neutralized with a solution of 8.4 g (0.1 mole) sodium bicarbonate in 200 ml of water. A solution of 0.2 moles sodium

benzylmercaptide in 200 ml of water (24.8 g benzylmercaptan neutralized with diluted sodium carbonate solution) was added cautiously with shaking. A voluminous white precipitate at once formed. This was filtered, washed with water and dried (20.2 g). M.p. 144–148°. Three recrystallizations from ethanol yielded pure substance with the melting point 152–153°.

Anal. Subst., 41.33 mg: CO_2, 92.62 mg; H_2O, 1956 mg.
6.39 mg: 7.40 ml of 0.00948 *N* NaOH.
62.32 mg: 8.87 ml of 0.00948 *N* NaOH.

$C_{38}H_{39}O_6S_4Na$ (743.0)
Calc. C 61.43; H 5.29; S 17.26; Na 3.10. Equ. wt. 743.0.
Found C 61.15; H 5.30; S 17.39; Na 2.96. Equ. wt. 741.5.

3,5-Dibenzylmercapto-levulinic acid

A white precipitate was obtained when the foregoing filtrate was acidified with dilute sulphuric acid. The precipitate was filtered, washed with water and airdried. The yield was 16 g (m.p. 102–110°). After three recrystallizations from benzene-cyclohexane pure, white needles were obtained. M.p. 115–116°5.

Anal. Subst., 57.82 mg: CO_2, 134.14; H_2O 2750 mg.
10.11 mg: 11.95 ml of 0.00948 *N* NaOH.
90.60 mg: 3.44 ml of 0.733 *N* NaOH.

$C_{19}H_{20}O_3S_2$ (360.5) Calc. C 63.30; H 5.59; S 17.79; Equ. wt. 360.5.
Found C 63.31; H 5.32; S 17.95; Equ. wt. 359.3.

The acid could also be liberated by treating the acid salt with dilute hydrochloric acid.

6-Benzylmercaptomethyl-3(2H)-pyridazinone

A. 10 g of 3,5-dibenzylmercapto-levulinic acid was treated with hydrazine hydrate according to Huang-Minlon (9). When the reaction was complete, the alkaline solution was diluted with water and extracted with ether. The ether solution gave on evaporation 1.3 g of solid product, m.p. 66–68°. After recrystallization from ethanol the melting point was 69.5–71°. A 1:1 mixture with an authentic specimen of dibenzyldisulfide (m.p. 70–71°) melted at 70–71°.

Acidification of the alkaline water solution gave 6 g of a greyish precipitate, m.p. 127–129°. Recrystallization from 90% ethanol gave large colorless plates, m.p. 131.5–132°. On crystallizing from water long colorless needles (m.p. 129–130°) were also obtained.

Anal. $C_{12}H_{12}OSN_2$ (232.3) Calc. C 62.04; H 5.21; S 13.80; N 12.06.
Found C 62.07; H 5.23; S 13.43; N 11.99.

B. The same compound was also prepared by heating 3,5-dibenzylmercapto-levulinic acid with excess of hydrazine hydrate, during two hours on a steam-bath. The solid reaction product was washed with ethanol, and benzylmercaptan and dibenzyldisulfide were extracted with ether. The residue consisted of quite pure 6-benzylmercaptomethyl-3(2H)-pyridazinone (m.p. 128–130°) in nearly quantitative yield.

Biological activity

The pyridazinone derivate was tested (serial dilution) against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus hemolyticus* and *Candida albicans* in nutrient broth and against a human strain of *Mycobacterium tuberculosis* in Dubos medium without and with 20% of horse serum. No inhibition of growth was observed with a concentration of 0.2 mg/ml.

The infrared absorption measurements

A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prism was used for the determinations. The substances were examined as solid pressed potassium bromide plates (13) or in liquid phase.

SUMMARY

Levulinic acid has been brominated in acetic acid giving 3,5-dibromolevulinic acid in a good yield. By treating the dibromo-acid with sodium benzylmercaptid, 3,5-dibenzylmercapto-levulinic acid and its acid sodium salt have been obtained. Attempts to reduce the carbonyl group of dibenzylmercapto-levulinic acid using Clemmensen and Wolff–Kishner reduction methods have not been successful. Instead of the expected substance, treatment with hydrazine hydrate gave a pyridazinone derivative the biological activity of which has been tested.

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